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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)	
)	
WALLER et al.)	Art Unit: 1644
)	
Application No. 09/945,339)	Examiner: Belyavskyi, Michail A.
)	
Filing Date: August 31, 2001)	Confirmation No. 1418
)	
For: METHODS OF TRANSPLANTATION)	
USING CHEMOTHERAPY-TREATED)	
ALLOGENEIC CELLS THAT ENHANCE)	
IMMUNE RESPONSE WITHOUT)	
GRAFT VERSUS HOST DISEASE)	

REPLY BRIEF

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

NEEDLE & ROSENBERG, P.C.
Customer Number 23859

Sir:

This is a substitute appeal brief submitted under 37 C.F.R. § 41.41 in reply to the Examiner's Answer mailed on June 9, 2006, which introduced new support for the rejections of record in response to an appeal from the final rejection of claims 1-6 and 15-20 in the Office Action mailed April 9, 2003. A Notice of Appeal was mailed on October 9, 2003.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is Emory University.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to appellants, the undersigned, or appellants' assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1-58 are pending. Claims 7-14 and 21-58 have been withdrawn from consideration as being drawn to a non-elected invention. Claims 1-6 and 15-20 stand rejected. Claims 1-6 and 15-20 are on appeal. The text of the claims on appeal are set forth in an appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

No amendments after final rejection have been filed.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

The present application solves a problem that has continually plagued transplants. That is, how to enhance transplant engraftment from matched unrelated or unmatched sibling donors without increasing the incidence of fatal graft versus host disease (GvHD), a disease that results when T cells, present in the graft, attack the graft recipient. The removal of T cells from the bone marrow results in a decreased incidence of graft vs. host reactions, but an increased incidence of rejection of the allogeneic bone marrow graft by the patient. Thus, lymphocytes, and especially T cells, present in the allogeneic bone marrow graft are important to ensure engraftment in antigenically and genetically mis-matched recipients. The claims on appeal are drawn to methods of reducing GvHD in a transplant recipient by administering to the recipient in combination with hematopoietic cells, mononuclear cells which are treated so as to substantially reduce their ability to cause GvHD while they retain their ability to proliferate. In particular, the claims on appeal focus on three features (1) treating mononuclear cells to reduce their ability to cause GvHD (which is described at least on page 1, line 16; page 6, lines 6-7; page 6, line 21; page 15, lines 18-19, and page 18, lines 19-20), (2) the treated cells retain the ability to proliferate (which is described at least on page 1, lines 16-17; page 6, line 7; page 6, lines 21-22; page 15, line 19, and page 18, line 20), and (3) the treated cells are administered to the transplant recipient (which is described at least on page 6, lines 4-6; page 6, lines 19-20; and page 15, lines 16-18). The dependent claims 2-4 and 16-18 refer to the type of mononuclear cells which may be treated. Such types of mononuclear cell are described at least on page 9, lines 10-21; and page 17, line 1 through page 18 line 17. Dependent claims 5, 6, 19, and 20 refer to the type of

treatment used on the mononuclear cells. Such treatments are described at least on page 9, lines 10-21 page 18, line 19 through page 19, line 17; and page 27, line 14 through page 28, line 2, where chemotherapeutic agents are discussed and specific agents are described.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

(i) Rejection of Claims 1-6 and 15-20 Under 35 U.S.C. § 103(a)

Claims 1-6 and 15-20 stand rejected under 35 U.S.C. § 103(a) as being obvious over U.S. Patent No. 5,800,539 to Waller ("Waller") in view of Sykes et al. (WO 99/25367; "Sykes"). Appellants respectfully traverse this rejection.

(7) ARGUMENTS

(i) Rejection of Claims 1-6 and 15-20 Under 35 U.S.C. § 103(a)

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1. The Issues

Appellants submit that the present rejection depends on the proper understanding of what the prior art discloses, the proper understanding of what the current claims require, the proper understanding of the law regarding 35 U.S.C. § 103(a) as it applies to the claimed methods, and a proper application of that law to the claimed methods. Appellants note that the Examiner has failed to achieve any of these goals in the present rejection.

The Examiner contends that the combination of Waller in view of Sykes renders the claimed invention obvious. In particular, the Examiner focuses on Sykes for its alleged disclosure of the ability of treated T cells to proliferate, which is not disclosed in Waller. The Examiner states that motivation for the combination would be that "one of skill in the art at the time the invention was made would deduce from the combined reference teaching that a treatment of donor T cells in such a way as to retain not only their viability as taught by Waller, but also their ability to proliferate in the recipient, as taught by Sykes, would be essential to successful engraftment of donor hematopoietic cells."

Appellants assert that (1) the combination of Waller and Sykes does not disclose or suggest the limitation of administering mononuclear cells treated so as to substantially reduce their ability cause graft versus host disease while they retain their ability to proliferate in the recipient, and (2) even if all the limitations were taught, the combination of Waller and Sykes is improper for the combination would change the principle of operation of Waller in that Waller requires the nonproliferation of T cells.

2. The Legal Standard

In order to establish *prima facie* obviousness of a claimed invention, three criteria must be met. First, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Second, there must be some suggestion or motivation to combine the references. Third, there must be a reasonable expectation of success. Also, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art. *In re Vaeck*, 947 F.2d. 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

In order for a claimed invention to be obvious, the invention as a whole must be considered, and in particular every limitation of the claim must be disclosed or suggested by the prior art. This means that for the present claims 1-6, the cited publications must disclose or suggest a method of transplanting hematopoietic cells from a donor source into a genetically unrelated recipient comprising administering to the recipient, in combination with the administration of the hematopoietic cells, an amount of mononuclear cells which are treated so as to substantially reduce their ability to cause graft versus host disease while they retain their ability to proliferate in the recipient and facilitate engraftment of the hematopoietic cells in the recipient; and administering to the recipient an effective amount of hematopoietic cells. For present claims 15-20, the cited publications must disclose or suggest a method of enhancing immune reconstitution in a transplant recipient, comprising administering to the recipient, in combination with a transplant, an amount of mononuclear cells which are treated so as to substantially reduce their ability to cause graft versus host disease while they retain their ability to proliferate in the recipient, and which are effective in enhancing immune reconstitution in the recipient. Appellants submit that the cited publications do not disclose or suggest all of these features.

Additionally, it has been established that the proposed modification or combination cannot change the principle of operation of the cited reference. That is, if a modification changes the principal operation of the prior art being modified, then the teachings are not sufficient to render the claims *prima facie* obvious. *In re Ratti*, 270 F.2d 810, 813, 123 USPQ 349 (CCPA 1959). This means that the modification of Waller through the combination of Sykes must not change the principal operation of Waller, which is the administration to the recipient, in combination with the administration of the hematopoietic cells, “an amount of mononuclear cells which are treated so as to render them incapable of proliferating and causing lethal graft versus host disease effect, but which are effective in enhancing subsequent engraftment of the hematopoietic cells in the recipient” (see, for example, Waller, column 3, lines 32-43). Appellants submit that the modification of Waller proposed in the rejection would require an impermissible change in the principle of operation of Waller’s method.

3. The Claims on Appeal

It has been recognized that T cells have both positive and negative effects when present in bone marrow transplant material. On the one hand, T cells are important for efficient engraftment of bone marrow cells in antigenically and genetically mismatched recipients. On the other hand, the presence of T cells in bone marrow transplants increases the incidence of graft versus host disease. In dealing with these effects, those performing bone marrow transplants have tried to balance removal and/or killing of T cells in bone marrow transplant material with retention of T cells in bone marrow transplant materials. Thus, the art recognized the presence or absence of T cells as being of significance to bone marrow transplants.

Appellants have discovered that it is not the mere presence or absence of T cells that matters in causing the positive and negative effects of T cells on bone marrow transplants. Specifically, Appellants have discovered that a reduction in the viability of T cells without eliminating or killing the T cells outright (that is, treating the T cells such that they retain their ability to proliferate) results in both the reduction of the negative effects of T cells on bone marrow transplants while retaining the positive effects. The present claims specifically claim treatment of mononuclear cells to obtain these benefits. Appellants submit that the cited publications do not disclose or suggest the claimed effects, do not disclose or suggest any way to obtain the claimed effects, and thus cannot make the present claims obvious.

The present claims are drawn to a method of transplanting hematopoietic cells from a donor source into a genetically unrelated recipient. To enhance the engraftment of the hematopoietic cells, mononuclear cells are administered with the transplanted hematopoietic cells. However, it has been recognized in the art that mononuclear cells also contribute to Graft versus Host Disease (GvHD). The claims overcome this problem by treating the mononuclear cells with an agent that reduces their ability to cause GvHD while maintaining their ability to proliferate. In particular, claims 1 and 15 recite that the mononuclear cells are “treated so as to substantially reduce their ability cause graft versus host disease while they retain their ability to proliferate in the recipient” (emphasis added). A careful reading of the claim language shows that the claims indicate that:

(A) the mononuclear cells are administered with the transplant (i.e., the hematopoietic cells from a donor source) and

(B) the mononuclear cells are “treated so as to substantially reduce their ability cause graft versus host disease while they retain their ability to proliferate in the recipient.”

Note that property (A) requires that the cells be treated prior to administration to the recipient of the transplant. This property is crucial since treating donor mononuclear cells after they have been administered to the recipient would affect all of the mononuclear cells in the recipient, both donor and host mononuclear cells, and could leave the patient further immunocompromised. By treating only those mononuclear cells to be administered with the hematopoietic cell transplant, only those cells that could cause versus host disease are affected.

4. Waller (U.S. Patent No. 5,800,539)

Waller discloses the administration to the recipient, in combination with the administration of the hematopoietic cells, an amount of mononuclear cells which are treated so as to render them incapable of proliferating and causing lethal graft versus host disease effect, but which are effective in enhancing subsequent engraftment of the hematopoietic cells in the recipient (see, for example, the abstract; column 3, lines 5-16; column 4, lines 40-48; column 4, line 66 to column 5, line 1; and claim 1). Note that Waller specifically discloses that the mononuclear cells should not proliferate. This is exactly the opposite of the claimed method. Not only does Waller fail to disclose proliferation of donor mononuclear cells, Waller specifically teaches away from proliferation of donor mononuclear cells. In particular, the

method of Waller requires the use of "mononuclear cells which are treated so as to render them incapable of proliferating" (see Waller, column 3, lines 32-43).

The Examiner's Answer of June 9, 2006, states that "the main principle operation of Waller is the ability of treated T cells to facilitate engraftment of the hematopoietic cells in the recipient, while not inducing lethal GvHD" (see Examiner's Answer page 7, paragraph 4). However, this disingenuously refers to two features of the method of Waller while leaving out a third required feature of Waller (that the mononuclear cells be incapable of proliferating). The fact that Waller specifies two features that the claims on appeal also makes use of does not alter the fact a third requirement of Waller is specifically excluded from the claims on appeal. Waller requires that the mononuclear cells be incapable of proliferation while the claims on appeal require that the mononuclear cells retain the ability to proliferate. To suggest or imply by omission (as the Examiner's Answer does) that a lack of proliferation is not integral to the process of Waller ignores the basis of how Waller describes that the results are to be achieved. In particular, on column 4, lines 44-48, Waller states that the "treatment sufficiently hinders the mononuclear cell proliferation such that they do not cause a lethal GvHD," thus linking the inability to proliferate required in Waller with the goal of Waller to prevent GvHD. Throughout Waller reference is made to the nonproliferative ability of the mononuclear cells being the characteristic that allows for engraftment, yet prevents GvHD (see, for example, column 3, lines 32-43; column 4, line 6 through column 5, line 15, where not only treatment to prevent proliferation is discussed, but treatment to induce eventual cell death is described; and column 5, lines 25-37). Thus, because Waller specifically discloses that proliferation should not occur, one of skill in the art would not be motivated to modify the method of Waller to require the proliferation of the mononuclear cells, as doing so would change the principle of operation of Waller.

5. Sykes et al. (WO 99/25367)

Sykes disclose the myeloreductive non-myeloablative treatment of mononuclear cells in the transplant recipient to reduce graft versus host disease while promoting graft versus leukemia (GVL) effects and promoting engraftment. Note that Sykes treats the recipient mononuclear population not an ex vivo donor mononuclear population, and the treatment is in the recipient, not ex vivo as required by the claims on appeal (see, for example, page 2, lines 6-11; page 2,

lines 18-26; and page 3, lines 1-12). The Examiner states that Sykes teaches that “non-depleted T cells help engraftment of donor hematopoietic cells,” that Sykes teaches “treatment with fludarabine,” and “that said treatment should not completely eliminate T cells” (see page 5, last paragraph of the Examiner’s Answer). However, the Examiner has simply confused the T cell populations being treated. In fact, the methods taught in Sykes affect an entirely different cell population for an entirely different purpose than the claims on appeal. In the claims on appeal, mononuclear cells from the donor are treated to prevent graft versus host disease. However, in Sykes, the recipient's own mononuclear cells are treated (that is, subjected to a non-myeloablative conditioning regimen) in preparation for the transplant to aid in the establishment of mixed chimerism (see, for example, page 13, lines 15-20; page 14, lines 5-16; and page 15, line 21 through page 17, line 31). The purpose of the establishment of a mixed chimera is to aid engraftment by preventing the anti-MHC responses associated with rejection of the graft. Therefore, contrary to the Examiner’s assertions on page 8, line 2 of the Examiner’s Answer, fludarabine treatment is not used to prevent GvHD, rather it is to deplete or inactivate the graft recipient's own T cells prior to transplantation so mixed chimerism can occur. Because this goal of Sykes is different from the goal of both Waller and the claims on appeal, Sykes does not suggest or support, and is inconsistent with, any modification of Waller. Thus, for at least these reasons, Sykes fails to supplement the failings of Waller.

Furthermore, Sykes describes treatments that do not completely deplete the T cells present. Sykes does not disclose or suggest that the cells have **retained the ability to proliferate**. Sykes is silent on the proliferative ability of the remaining T cells. Thus, contrary to assertions in the rejection, Sykes does not disclose or suggest that the T cells retain the ability to proliferate.

Even if Sykes did encompass the treatment of donor T cells (it does not) and did encompass the use of treated donor T cells that retained the ability to proliferate (it does not), that would not make the present rejection proper. In this regard, Appellants note that art that encompasses (among other possibilities) a particular feature, but which does not disclose that particular feature, does not put those of skill in the art in possession of that feature. For example, art disclosing an alloy comprising some nickel does not disclose or make obvious an alloy comprising enough nickel to give the alloy a particular hardness. Until it is discovered that such

an effect is possible and that such an amount of nickel is desirable, this particular alloy is unknown and unobvious to those in the art. The situation here is analogous. It is the Appellants who discovered the importance of the claimed treatment and features. None of the cited publications disclose or suggest treatment to obtain the claimed effects.

6. Combination of Waller and Sykes

The failings of the present rejection can be simply summarized. The claims require *ex vivo* treatment of mononuclear cells (that is, treatment prior to their administration to a recipient) and require that the treated mononuclear cells retain the ability to proliferate. First, neither Waller nor Sykes disclose or suggest that administered mononuclear cells retain the ability to proliferate. In fact, Waller specifically requires that the administered mononuclear cells be incapable of proliferating. Thus, neither of the cited publications disclose retention of the ability to proliferate. This alone is fatal to the rejection. Second, Sykes discloses treatment of the recipient patient, not *ex vivo* treatment of cells. Thus, the treatment disclosed by Sykes is not clearly relevant either to the claims on appeal or to Waller, and those of skill in the art would not be motivated to apply Sykes to the method of Waller. Finally, even if Sykes suggested *ex vivo* treatment of mononuclear cells such that they retain the ability to proliferate (it does not), this could not be applied to the method of Waller because to do so would result in the impermissible change in the principle of operation of the method of Waller (that is, such modification would require exactly the opposite state of proliferative ability than is required by Waller).

As discussed above, neither the Waller nor Sykes disclose or suggest the claimed invention. In fact in the Office Action mailed November 18, 2002, concedes that Waller does not disclose or suggest that the treated T cells retain their ability to proliferate in the recipient. The April 9, 2003 Office Action states that

Sykes et al., teach a method of myeloreductive non-myeloablative treatment with fludarabine, the same type of treatment as [the] claimed invention. Sykes et al., teach that for successful transplantation of hematopoietic cells from donor to recipient, it is essential that after treatment T cells are not completely depleted, thus so called graft-versus-leukemia (GvL) effects of the non-depleted T cells help engraftment of donor hematopoietic cells (see page 10, lines 17-23, page 11, lines 5-25 in particular). Sykes et al., specifically stressed that said

treatment should not completely eliminate T cells (page 16, lines 2-11 in particular).

Appellants respectfully point out that none of this establishes that Sykes discloses or suggests that the T cells retain the ability to proliferate nor that the T cells are to be treated ex vivo. Sykes is silent as to proliferation, and Waller requires non-proliferation. The rejection argues that because the claimed method results in mononuclear cells that retain the ability to proliferate, an allegedly similar treatment by Sykes must inherently result in T cells that retain the ability to proliferate. This reasoning is incorrect. First, Appellants' invention cannot be used to provide what is missing from the art (that is, retention of proliferative ability). Second, the method of Sykes does not inherently result in the T cells that retain the ability to proliferate. In fact, in the absence of Appellants' invention, Waller indicates to those of ordinary skill in the art that a similar treatment (such as treatment with fludarabine) would result in cells that lack the ability to proliferate. It is not seen how the cited publications come close to providing the critical limitation of retention of the ability to proliferate required by the claims on appeal. Third, a merely possible, but undisclosed, property of a prior art composition does not meet the legal standard for inherency. An inherent property must necessarily be present in the prior art, not merely a possibility. "The mere fact that a certain thing may result from a given set of circumstances is not sufficient." *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999). Thus, even if the T cells of Sykes might possibly retain the ability to proliferate, that property cannot be inherent in the cells because it would not necessarily be present. Waller proves that retention of the ability to proliferate is not a necessary outcome of a treatment such as that of Sykes. For all of these reasons, the rationale of the rejection cannot be accepted. As a result, the present rejection fails and should be reversed.

Waller discloses a method of preventing graft-versus-host disease comprising treatment with fludarabine (see column 3, lines 6-16, which discuss the treatment, and column 4, line 66 through column 5, line 12, for the use of fludarabine, in particular). Waller also discloses that "lymphocytes, and especially T cells, present in the allogeneic bone marrow graft are important to ensure engraftment" (column 1, lines 52-55). Waller goes on to state that "T cells present in the allogeneic graft also have an important role in eliminating residual cancer cells in the recipient, a phenomenon termed "graft vs. leukemia effect" (column 1, lines 55-58). However,

Waller is clear that the T cells are treated so as to render them incapable of proliferation (column 3, lines 6-16, column 5, lines 25-31, and claims 1 and 2).

Thus, both Waller and Sykes use fludarabine to reduce T cell populations. Further, only Waller discloses an effect of this use of fludarabine on the proliferative ability of the treated T cells (the proliferative ability is eliminated). In the face of this, it cannot be said that Sykes discloses (or is even consistent with the possibility) that the cells of Sykes retain the ability to proliferate. Accordingly, it would not be obvious to one of skill in the art to use fludarabine to result in T cells capable of proliferating.

The Examiner's statements on page 8 of the Examiner's Answer regarding the ability of the claimed method to result in T cells retaining the ability to proliferate and that the concentration of cytotoxic drug needed to reduce the ability of treated cells to cause GvHD "is that which causes greater than 90% inhibition of proliferation of treated cells," are incorrect and only serve to distract from the real legal issue. Regarding the ability of treated cells to proliferate, the Examiner states that "there is not any evidence in the specification that the claimed ability of treated mononuclear cells to proliferate ...has ever been checked." However, page 34 of the instant application clearly shows that treated cells were checked for proliferative ability and provides evidence that the cells did proliferate. Thus, the Examiner's statements are incorrect and thus cannot support the Examiner's conclusion. Moreover, focusing on the reduced proliferative ability over non-treated cells improperly ignores the simple observation that the treated cells do retain the ability to proliferate. Further, the proliferative ability of the cells in the example is not at issue and is not relevant to the rejection because the claims on appeal require the ability to proliferate. Finally, focusing on these areas fails to address the fact that Waller explicitly teaches the non-proliferation of treated cells and the modification of Waller to accommodate proliferation would result in an impermissible change in the principle operation of Waller.

Additionally, while Sykes does disclose that T cells should not be completely depleted, this is not the same as saying that the remaining cells would retain the ability to proliferate nor that such a characteristic would be desirable. The presence or absence of T cells in the recipient is completely independent of their ability to proliferate. The art is replete with examples of non-proliferating T cells (see, for example, Jenkins MK, Schwartz, RH. (1987) *J. Exp Med.* 165:302-

19; Jenkins MK, et al. (1987) *Proc Natl. Acad. Sci.* 84:5409-13; Quill H, Schwartz, RH. (1987) *J. Immunol.* 138:3704-12). Appellants respectfully contend that the rejection extrapolates an effect that is not discussed anywhere in Sykes.

Furthermore, the rejection implies, in error, that any treatment with fludarabine would result in proliferating T cells--since that is what is presently claimed--and that Sykes intended that the T cells proliferate. This is incorrect. In fact, and to the contrary, Sykes discloses that "in preferred embodiments, immune cell activity, e.g., T cell activity, preferably graft reactive T cell activity, is inhibited in the subject" (page 14, lines 26-31). By this, Sykes means that the number of T cells is reduced. This is made clear where Sykes defines the term "immune cell activity" as "reducing the number of active immune cells, e.g., thymocytes, T cells...in a subject. Inhibition can include partial inhibition or partial reduction (as opposed to total elimination) of the number of active immune cells, e.g., T cells" (page 10, lines 18-22; emphasis added). This definition emphasizes reduction in the number of cells, not in any change in cell characteristics. Thus it is clear that Sykes viewed treatment with fludarabine as a means to reduce the T cell population not to maintain the proliferative capacity of the T cells. This view of Sykes is further supported by embodiments that disclose "immunosuppression regimen for suppressing or depleting T cells in the transplanted donor stem cells" (page 5, lines 21-23, page 5, lines 31-33, page 21, lines 6-7, and page 21, lines 16-17), and by the statement (on page 2 lines 15-20) "[l]ikewise, the method can include the further step of treating the subject with an immunosuppressant regimen, after introduction of the donor stem cells....[s]uch immunosuppressants can include independently of pre- and post-transplantation is [sic] both are carried out, a treatment of the subject which inactivates and/or depletes host T lymphocytes." If the goal, as indicated, is depletion, then surely the depleted cells cannot be expected to proliferate. Furthermore, it is clear throughout Sykes and at least on page 15, lines 24-32, that in addition to donor derived T cells, host T cells are also to be depleted. Thus, it is clear that Sykes does not disclose or suggest the use of fludarabine to enable T cells to proliferate, but to the contrary discusses fludarabine only in the context of immunosuppression. For at least these reasons, the combination of Waller with Sykes does not make the claims obvious.

Moreover, it is clear that not all fludarabine treatments would result in a reduced T cell population that retains its proliferative capacity. The art is replete with examples of treatments

with fludarabine that resulted in nonproliferative T cells. Waller is an example. Numerous publications in the area use fludarabine to eliminate T cells. Goodman et al., (1996) *Am. Surg.* 62(6):435-442, states that “[f]ludarabine phosphate selectively eliminates normal and malignant mononuclear cells in large animals and man.” Additionally Goodman et al. report that “[t]he drug depletes mononuclear cells from culture within 24 hours of initial exposure, CD4 and CD8 T cells being more sensitive than either CD20 B cells or CD34 bone marrow precursors.” Additionally, Boulad et al., (2000) *Br. J. Haematol.* 111(4):1153-7, discusses fludarabine-based cytoreductive treatment in a subject with Fanconi anaemia. Contemporary with Sykes and Waller, the art of hematopoietic stem cell transfers was filled with publications detailing the importance of reducing or depleting T cell populations to prevent graft versus host disease, not retaining T cells (see for example; Link, (1999) *Baillieres Best Pract Res Clin Haematol.* 12(1-2):87-98, and Slaper-Cortenbach, ICM, et al., (1999) *Rheumatology* 38:751-754). For at least these reasons, the combination of Waller with Sykes does not make claims obvious.

The rejection cites the paragraph on page 10, lines 17-23, of Sykes, which discusses the definition of “inhibiting immune cell activity,” referring, in particular, to the last sentence which states “[i]nhibition can include partial inhibition or partial reduction (as opposed to total elimination) of the number of active immune cells e.g., T cells.” The Examiner reads this to mean that total elimination is not desired (and thus, impliedly, that proliferation is desirable). However, the more reasonable reading of this passage is not that total elimination is undesirable, but rather a recognition that a small residual population of T cells would likely remain following treatment and therefore the Sykes specification was written to reflect that T cells may remain after treatment. This passage does not refer to the proliferative capacity of the T cells. As such, Appellants submit that this passage does not support the Examiner’s position.

7. Summary and Conclusions

Appellants have established that the claimed method is not obvious over Waller in view of Sykes. In particular, Appellants have established that (1) Waller and Sykes do not disclose or suggest a method of administering mononuclear cells treated so as to substantially reduce their ability cause graft versus host disease while they retain their ability to proliferate in the recipient; (2) retention of proliferative ability is not inherent in the method of Sykes and thus is not present

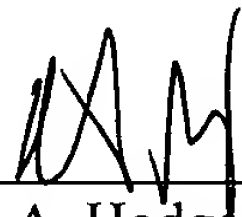
in the cited art, (3) the combination of Waller and Sykes would change the principle of operation of Waller and therefore cannot be used to establish a *prima facie* case of obviousness.

For the foregoing reasons, Appellants submit that the claims 1-6 and 15-20 are patentable and request reversal of the rejections.

No fee is believed to be due; however, the Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.

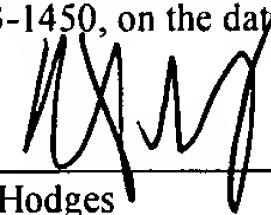


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Robert A. Hodges

Date

8/9/2006

(8) CLAIMS APPENDIX

1. A method of transplanting hematopoietic cells from a donor source into a genetically unrelated recipient, comprising:
 - a) administering to the recipient, in combination with the administration of the hematopoietic cells, an amount of mononuclear cells which are treated so as to substantially reduce their ability to cause graft versus host disease while they retain their ability to proliferate in the recipient and facilitate engraftment of the hematopoietic cells in the recipient; and
 - b) administering to the recipient an effective amount of hematopoietic cells.
2. The method of claim 1, wherein the mononuclear cells are T cells.
3. The method of claim 1, wherein the mononuclear cells are natural killer cells.
4. The method of claim 1, wherein the mononuclear cells are a mixture of T cells and natural killer cells.
5. The method of claim 1, wherein the cells are treated with a chemotherapeutic agent.
6. The method of claim 5, wherein the chemotherapeutic agent is selected from the group consisting of 9-D-arabinofuranosyl-2-fluoroadenosinemonophosphate (fludarabine), 2'-deoxycytidine (pentostatin), 2-chlorodeoxyadenosine (2CDA), 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), 2'-deoxy-2', 2'-difluorocytidine (gemcitabine) and 2-amino-9-D-arabinosyl-6-methoxy-9-H-purine (Ara-G, 506U78).
15. A method of enhancing immune reconstitution in a transplant recipient, comprising administering to the recipient, in combination with a transplant, an amount of mononuclear cells which are treated so as to substantially reduce their ability to cause graft versus host disease while they retain their ability to proliferate in the recipient, and which are effective in enhancing immune reconstitution in the recipient.
16. The method of claim 15, wherein the mononuclear cells are T cells.
17. The method of claim 15, wherein the mononuclear cells are natural killer cells.

18. The method of claim 15, wherein the mononuclear cells are a mixture of T cells and natural killer cells.
19. The method of claim 15, wherein the cells are treated with a chemotherapeutic agent.
20. The method of claim 19, wherein the chemotherapeutic agent is selected from the group consisting of 9-D-arabinofuranosyl-2-fluoroadenosinemonophosphate (fludarabine), 2'-deoxcoformycin (pentostatin), 2-chlorodeoxyadenosine (2CDA), 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), 2'-deoxy-2', 2'-difluorocytidine (gemcitabine) and 2-amino-9-D-arabinosyl-6-methoxy-9-H-purine (Ara-G, 506U78).

(9) EVIDENCE APPENDIX

List of Evidence Involved in Appeal

1. Jenkins MK, Schwartz, RH. (1987) *J. Exp Med.* 165:302-19
2. Jenkins MK, et al. (1987) *Proc Natl. Acad. Sci.* 84:5409-13
3. Quill H, Schwartz, RH. (1987) *J. Immunol.* 138:3704-12
4. Goodman et al., (1996) *Am. Surg.* 62(6):435-442
5. Boulad et al., (2000) *Br. J. Haematol.* 111(4):1153-7
6. Link, (1999) *Baillieres Best Pract Res Clin Haematol.* 12(1-2):87-98
7. U.S. Patent No. 5,800,539 to Waller
8. Sykes et al. (WO 99/25367
9. Slaper-Cortenbach, ICM, et al., (1999) *Rheumatology* 38:751-754

Statement of Entry in the Record

Appellants submit herewith copies of evidence for use in the appeal. Documents 1-6 and 9 (Jenkins, Jenkins, Quill, Goodman, Boulad, Link, and Slaper-Cortenbach) were submitted by Appellants in the August 11, 2003 response to the April 9, 2003 Final Office Action. Documents 7 and 8 (U.S. Patent No. 5,800,539 and Sykes) were first entered into the record by the Examiner in the Office Action mailed on November 18, 2002

(10) RELATED PROCEEDINGS APPENDIX

None

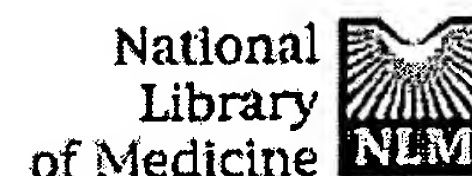
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Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo.

Jenkins MK, Schwartz RH.

We investigated the antigen specificity and presentation requirements for inactivation of T lymphocytes in vitro and in vivo. In vitro studies revealed that splenocytes treated with the crosslinker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) and soluble antigen fragments failed to stimulate significant proliferation by normal pigeon cytochrome c-specific T cell clones, suggesting that the chemical treatment inactivated full antigen presentation function. However, T cell clones exposed to ECDI-treated splenocytes and antigen in vitro were rendered unresponsive for at least 8 d to subsequent antigen stimulation with normal presenting cells. As predicted by the in vitro results, specific T cell unresponsiveness was also induced in vivo in B10.A mice injected intravenously with B10.A, but not B10.A(4R), splenocytes coupled with pigeon cytochrome c via ECDI. The antigen and MHC specificity of the induction of this T cell unresponsiveness in vitro and in vivo was identical to that required for T cell activation. These results suggest that nonmitogenic T cell recognition of antigen/MHC on ECDI-modified APCs results in the functional inactivation of T cell clones.

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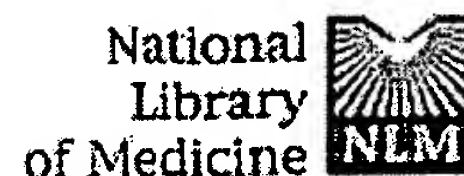
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Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones.

Jenkins MK, Pardoll DM, Mizuguchi J, Chused TM, Schwartz RH.

Exposure of normal interleukin 2 (IL-2)-producing helper T-cell clones to antigen and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-treated antigen-presenting cells results in proliferative unresponsiveness to subsequent stimulation with antigen and normal antigen-presenting cells. In the present study, we have examined the molecular events that accompany the induction of this unresponsive state. T cells stimulated in this manner failed to produce IL-2, but interleukin 3, interferon-gamma, and IL-2 receptors were partially induced and T-cell receptor beta mRNA was fully induced. Although T-cell unresponsiveness correlated with an IL-2 production defect, addition of IL-2 during the induction phase failed to prevent development of the unresponsive state. The critical biochemical event appeared to be an increase in intracellular calcium. Removal of calcium from the medium prevented induction of the unresponsive state, whereas addition of the calcium ionophore ionomycin induced unresponsiveness as well as all of the related partial activation events. Thus, an increase in intracellular calcium under nonmitogenic conditions appears to initiate an alternative activation program that prevents the T cell from producing IL-2 in response to subsequent normal activation signals. The significance of this in vitro model for tolerance induction in vivo is discussed.

PMID: 2955418 [PubMed - indexed for MEDLINE]

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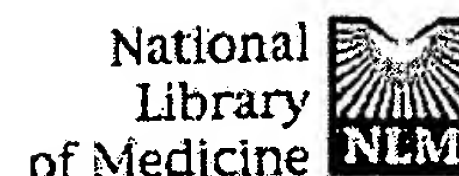
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Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness.

Quill H, Schwartz RH.

Culture of normal inducer T cell clones with antigen and purified Ek beta:Ek alpha incorporated into planar lipid membranes resulted in specific T cell activation as determined by cell volume increase and IL 3 production. However, in contrast to results obtained with T cell hybridomas, antigen presentation by planar membranes did not induce measurable IL 2 production, and proliferative responses were not detected. Rather, recognition of only Ek beta:Ek alpha and antigen resulted in the specific induction of a long-lived state of proliferative nonresponsiveness to subsequent stimulation by conventional APC and antigen. Induction of nonresponsiveness required protein synthesis, and was not simply due to the absence of IL 2. The antigen-nonresponsive cells could respond to either PMA plus ionomycin or IL 2, and they expressed normal levels of surface antigen-receptor molecules. These results demonstrate that recognition by normal T cell clones of antigen and Ia molecules in the absence of other accessory cell molecules and signals results in a prolonged state of proliferative nonresponsiveness, possibly similar to a state of T cell tolerance in vivo.

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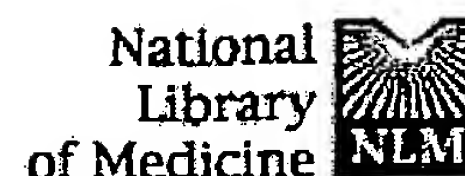
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Fludarabine phosphate: A DNA synthesis inhibitor with potent immunosuppressive activity and minimal clinical toxicity.

Goodman ER, Fiedor PS, Fein S, Athan E, Hardy MA.

Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, USA.

Fludarabine phosphate selectively eliminates normal and malignant mononuclear cells in large animals and man through the inhibition of DNA synthesis. The drug depletes mononuclear cells from culture within 24 hours of initial exposure, CD4 and CD8 T cells being more sensitive than either CD20 B cells or CD34 bone marrow precursors. Mitogenic activation of lymphocytes enhances cellular elimination from culture. Fludarabine inhibits PHA-induced T-cell proliferation by >90 per cent and mixed lymphocyte reactions (allogeneic and xenogeneic) by >95 per cent. Fludarabine exerts its cytolytic effects through the induction of endonuclease-independent apoptosis. A 5-day course of fludarabine (50 mg/m² intravenously once daily) induces both T- and B-cell lymphopenia in Cynomolgus monkeys and Papio baboons. Transient neutropenia was the only side-effect seen in experimental animals. Pretreatment of Cynomolgus monkeys with this regimen of fludarabine caused a prolongation of ABO-compatible skin allograft survival from 8 days (control) to 16 days (drug treated group). Secondary allotransplantation into presensitized recipients showed a similar prolongation of graft survival with fludarabine pretreatment (8 days vs 5 days control). Fludarabine promises to be a potent immunosuppressive agent with low clinical toxicity.

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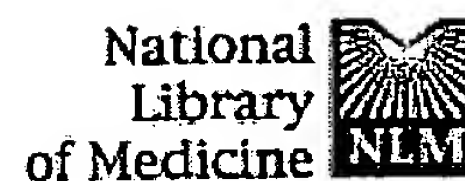
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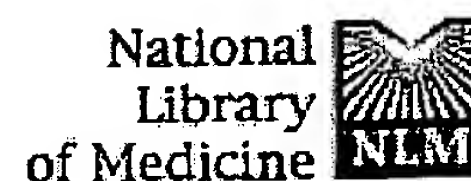
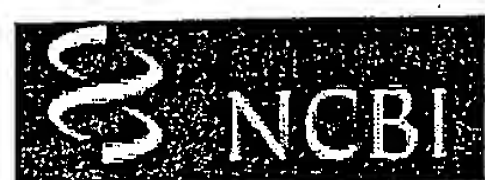
Stem cell transplantation for the treatment of Fanconi anaemia using a fludarabine-based cytoreductive regimen and T-cell-depleted related HLA-mismatched peripheral blood stem cell grafts.

Boulad F, Gillio A, Small TN, George D, Prasad V, Torok-Castanza J, Regan AD, Collins N, Auerbach AD, Kernan NA, O'Reilly RJ.

Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.
bouladf@mskcc.org

We have employed a new cytoreductive regimen to transplant two patients with Fanconi anaemia (FA), using T cell-depleted two HLA-allele disparate related peripheral blood stem cell transplants (PBSCTs). Patient 1, a 5-year-old male with FA and aplastic anaemia, initially received an HLA two-antigen mismatched unrelated cord blood transplant and failed to engraft. He received fludarabine (Flu) and cyclophosphamide (Cy), followed by a CD34(+) E-rosette(-) (CD34(+)E(-)), T cell-depleted, granulocyte colony-stimulating factor (G-CSF)-mobilized PBSCT from his HLA B-DRB1 mismatched father. He received anti-thymocyte globulin (ATG), steroids, FK506 and G-CSF after transplant for rejection and graft-versus-host disease (GVHD) prophylaxis. The patient is now 23 months after SCT with no evidence of GVHD and with full haematopoietic and immune reconstitution. Patient 2, a 10-year-old boy with FA and myelodysplastic syndrome, received single-dose total body irradiation (SDTBI), Flu and Cy followed by a CD34(+)E(-), T-cell-depleted G-CSF-mobilized PBSCT from his HLA B-DRB1 mismatched sister. He also received ATG, steroids, FK506 and G-CSF after transplant. The patient is now 12 months after SCT in complete remission with no evidence of GVHD. Absolute neutrophil counts (ANC) of $> 1 \times 10^9/l$ were achieved on day 11 and day 10 post transplant respectively. Both patients are fully engrafted. In summary, we report two successful T-cell-depleted stem cell transplants from mismatched related donors for the treatment of Fanconi anaemia, using a fludarabine-based cytoreduction. Both patients experienced minimal toxicity, rapid engraftment and no GVHD.

PMID: 11167755 [PubMed - indexed for MEDLINE]



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1: Baillieres Best Pract Res Clin Haematol. 1999 Mar-Jun;12(1-2):87-98.

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T-cell depletion of allogeneic peripheral blood stem cells.

Link H.

Department of Internal Medicine I, Westfalz Medical Centre, Kaiserslautern Germany.

The high content of immunocompetent T-cells in apheresis products may expose recipients of allogeneic peripheral blood stem cells (PBSC) to an elevated risk of acute and chronic graft-versus-host disease (GvHD). Thus, the use of an appropriate T-cell reduction or depletion technique might reduce this risk. The hazards of rejection and of a higher relapse rate should be avoided by maintaining a portion of the T-cells in the graft or by increasing the number of transplanted stem cells. The positive selection of CD34+ cells from peripheral blood preparations simultaneously provides an approximately 1,000-fold reduction of T-cells. Purified CD34+ cells containing committed and pluripotent stem cells are suitable for allogeneic transplantation. In transplantation from HLA-mismatched or three HLA-loci different family donors the amount of stem cells can be increased for reducing the incidence of rejection without increasing the T-cell number. In cases of poor marrow graft function a 'boost' with stem cells from the same family donor can be given. The risk of GvHD in transplantation from volunteer-matched unrelated donors might be reduced by T-cell depletion. If T-cells are used for enhancing the graft-versus-leukaemia effect, CD34+ enriched cells can be given for haematopoietic engraftment.

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		(43) International Publication Date: 27 May 1999 (27.05.99)
(21) International Application Number: PCT/US98/24209 (22) International Filing Date: 13 November 1998 (13.11.98) (30) Priority Data: 60/073,230 14 November 1997 (14.11.97) US (71) Applicant (for all designated States except US): THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SYKES, Megan [US/US]; 197 Eighth Street #301, Boston, MA 02129 (US). SPITZER, Thomas, R. [US/US]; 14 Seten Circle, Andover, MA 01810 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: TREATMENT OF HEMATOLOGIC DISORDERS (57) Abstract The inventors have discovered that hematologic disorders, e.g., both neoplastic (hematologic cancers) and non-neoplastic conditions, can be treated by the induction of mixed chimerism using myeloreductive, but not myeloablative, conditioning. Methods of the invention reduce GVHD, especially GVHD associated with mismatched allogeneic or xenogeneic donor tissue, yet provide, for example, significant graft-versus-leukemia (GVL) effect and the like.		

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Treatment of Hematologic Disorders

Government Funding

5 The work herein was supported by a grant from the National Institutes of Health. The government may have certain rights in the invention.

Background of the Invention

10 The invention relates to the treatment of hematologic disorders, e.g., disorders characterized by unwanted cells of hematopoietic origin, e.g., hematologic cancers.

15 Bone marrow transplantation (BMT) has yet to realize its full potential for the treatment of hematologic malignancies. A major obstacle to further advancement is graft-versus-host disease (GVHD), which has been prevented by removing T cells from the donor marrow. Unfortunately, T cell depletion has been associated with increased rates of engraftment failure and leukemic relapse. Despite improvements in pharmacologic GVHD prophylaxis, severe acute and chronic GVHD are still major complications of HLA-matched sibling bone marrow transplantation. Immunosuppressive drugs used for GVHD prophylaxis may also increase the relapse rate for certain types of leukemia. The patients receiving allogeneic BMT are, nevertheless, a fortunate select group: most patients do not have an HLA-matched sibling or a phenotypically matched unrelated donor, and therefore do not have the option of BMT. Attempts to perform BMT between strongly HLA-mismatched donor-recipient pairs have been associated with a prohibitively high incidence of severe GVHD and of failure of engraftment. Furthermore, a large fraction of leukemias and lymphomas afflict older patients who are more prone to the development of GVHD than are younger persons, and who therefore are not generally considered candidates for BMT, despite the lack of other curative options.

Summary of the Invention

30 The inventors have discovered that hematologic disorders, e.g., both neoplastic (hematologic cancers) and non-neoplastic conditions, can be treated by the induction of mixed chimerism in the absence of whole body irradiation (total myeloablation protocols) or other myeloablative treatment. Methods of the invention reduce GVHD,

especially GVHD associated with mismatched allogeneic or xenogeneic donor tissue, yet provide significant graft-versus-leukemia (GVL) effect and the like.

Certain embodiments of the subject methods also feature preparative regimens which minimize or eliminate the need for myeloablative treatment, e.g., hematopoietic space-creating irradiation, especially, preparative whole body irradiation.

One aspect of the present invention provides a method for treating a subject having a hematologic disorder comprising: (i) administering a myeloreductive non-myeloablative treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (ii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form chimeric bone marrow in the subject.

In certain embodiments, the myeloreductive treatment includes treating the subject with an immunosuppressant regimen, prior to introduction of the donor stem cells, in an amount sufficient to prevent rejection of the donor stem cells.

Likewise, the method can include a further step of treating the subject with an immunosuppressant regimen, after introduction of the donor stem cells, in an amount sufficient to prevent a graft-versus-host response mediated by the donor stem cells.

Such immunosuppressant regimens can include, independently for pre- and post-transplantation is both are carried out, a treatment of the subject which inactivates and/or depletes host T-lymphocytes and/or natural killer (NK) cells in the subject. For example, the immunosuppressant regimen includes treatment with T cell-depleting anti-CD4 and/or CD8 antibodies, such as anti-thymocyte globulin (ATG), OKT3 (Orthoclone OKT3 monoclonal antibody, Ortho Pharmaceutical Corp), LO-CD2a (US Patent 5,730,979), or Minnesota anti-lymphoblast globulin (MALG). Preferably, the immunosuppressant regimen, both before and after transplantation, includes administration of ATG.

Moreover, the immunosuppressant regimen can include treatment with thymic irradiation. Preferably, the pre-transplantation immunosuppressant conditioning includes administration of ATG and thymic irradiation.

In other embodiments, the immunosuppressant regimen includes treatment with one or more of a macrolide immunosuppressant, azathioprine, steroids (e.g., prednisone, methyl prednisolone), sub-lethal nonmyeloablative irradiation of lymphocyte-containing tissue, or costimulatory blocking agents (e.g., anti-CD40 ligands, CTLA4Ig fusion proteins, see, e.g., Lenschow et al., (1992) *Science* 257:789; and Turka et al., (1992) *PNAS* 89:11102).

In certain embodiments, the myeloreductive treatment includes treating the subject, prior to introduction of the donor stem cells, with a cyto-reductive agent selected from one or more of alkylating agents (e.g., nitrogen mustards [such as mechlorethamine], cyclophosphamide, melphalan and chlorambucil), alkyl sulphonates (e.g., busulphan), nitrosoureas (e.g., carmustine, lomustine, semustine and streptozocine), triazines (e.g., dacarbazine), antimetabolites (e.g., folic acid analogs such as methotrexate), pyrimidine analogs (e.g. fluorouracil and cytarabine), purine analogs (e.g., fludarabine, idarubicin, cytosine arabinoside, mercaptopurine and thioguanine), vinca alkaloids (e.g., vinblastine, vincristine and vendesine), epipodophyllotoxins (e.g., etoposide and teniposide), antibiotics* (e.g., dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin and mitomycin), dibromomannitol, deoxyspergualine, dimethyl myleran and thiotepa.

Preferably, the myeloreductive treatment includes treating the subject with cyclophosphamide.

Preferably, the pre-transplantation conditioning includes administration of ATG and cyclophosphamide, and thymic irradiation. Preferably the cyclophosphamide, or other cyto-reductive agents, are substantially cleared from the patient so as not inhibit proliferation of the transplanted stem cells.

An important use of the subject method is for allogeneic transplantation of donor stem cells which are mismatched, with respect to the subject, at one or more HLA class II antigens.

Another important use of the subject method is for allogeneic transplantation of donor stem cells which are mismatched, with respect to the subject, at two or more HLA antigens (either HLA class I or II or both).

In preferred embodiments, the donor stem cells are provided as allogeneic bone marrow, mobilized peripheral blood cells, or cord blood cells.

The donor stem cells, in some instances, can be expanded *ex vivo* for transplantation.

In preferred embodiments, the donor stem cells are from the same species as the subject. However, the present application also specifically contemplates that the donor stem cells are xenogeneic stem cells from a different species than the subject. In xenogeneic methods, the subject is a mammal, preferably a primate and more preferably a human. The donor mammal can be, by way of example, a swine, e.g., a miniature swine, or a nonhuman primate. In xenogeneic methods the donor of stem cells and the donor of leukocytes need not be the same individual but can be from different

individuals which are MHC matched or highly inbred, e.g., inbred miniature swine which are MHC matched.

In preferred embodiments, the subject is a human, and even more preferably, the subject is a human and donor stem cells are from another human.

5 The methods of the present invention can be used to treat a wide range of hematologic disorders, including neoplastic proliferation of hematopoietic cells, such as lymphoblastic leukemia, myelogenous leukemia, Hodgkin's lymphoma, Non-Hodgkin's lymphoma and myelodysplastic syndrome. As described herein, the subject method can be used to treat hematologic disorders which are refractory to chemotherapy, such a
10 chemorefractory Non-Hodgkin's lymphoma.

In other embodiments, the subject method can be used to treat hematologic disorders which are non-malignant, such as erythrocyte abnormalities or immune system disorders. For example, the instant method can be used to treat hemoglobinopathies, e.g., sickle cell anemia, aplastic anemia or thalassemia. The subject method also can be
15 used as part of a treatment regimen for autoimmune disorders as well as immunodeficiencies.

In several embodiments, particularly where little, and preferably no GVHD is detected post-transplantation (e.g., at least 14 days, and more preferably at least 25, 30 or even 35 days), the subject method includes the further step of administering allogeneic
20 donor leukocytes to the subject after introduction of the donor stem cells. The administration of donor leukocytes should be delayed sufficiently from the time of any hematopoietic space creating treatment such that the level of pro-inflammatory cytokines induced by the space creating treatment has subsided sufficiently to reduce or substantially eliminate GVHD from the donor leukocytes.

25 The subject method can also include the management of GVHD responses post-transplantation by administration of immunosuppressants, or by use of engineered stem cells which give rise to small molecule ablatable T cells or other hematopoietic cells. See, for example, U.S. Patent No. 5,834,266.

In another aspect, the invention features a method of treating a non-neoplastic
30 disorder or a hemoglobinopathy, e.g., sickle cell anemia, aplastic anemia, thalassemia

Thus, in one preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (iii) introducing into the subject, allogeneic donor
35 hematopoietic stem cells (donor stem cells) to form stable mixed chimeric bone marrow

in the subject.

In another preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism
5 can be induced in the subject, and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, wherein the donor stem cells are mismatched, with respect to the patient, at one or more class II HLA antigens.

In still another preferred embodiment, the subject method comprises: (i)
10 identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed
15 chimeric bone marrow in the subject, wherein the donor stem cells are mismatched, with respect to the patient, at two or more HLA antigens, e.g., class I and/or class II.

In yet another preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, (iii) introducing into the subject,
20 allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, and (iv) administering a post-transplantation immunosuppression regimen for suppressing or depleting T-cells in the transplanted donor stem cells.

In yet another preferred embodiment, the subject method comprises: (i)
25 identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a pre-transplantation conditioning to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, which pre-transplantation conditioning includes treating the cells with cyclophosphamide, ATG and thymic irradiation in an amount sufficient to reduce rejection of transplanted donor stem cells;
30 and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, and (iv) administering ATG to the subject post-transplant for suppressing or depleting T-cells in the transplanted donor stem cells.

Another aspect of the present invention relates to the use of donor allogeneic
35 stem cells in the manufacture of a medicament for the treatment of a hematologic

disorder, wherein the medicament administered to a patient conditioned with myeloreductive non-myeloablative treatment, and in an amount sufficient to form chimeric bone marrow in the subject.

Still another aspect of the present invention provides a kit for allogeneic hematopoietic stem cell transplantation. The kit includes cyclophosphamide in an amount sufficient to reduce rejection of transplanted donor stem cells when administered to a patient pre-transplantation, and ATG in an amount sufficient to reduce rejection of transplanted donor stem cells when administered to a patient pre-transplantation and suppress T-cells in transplanted donor stem cells. The kit may also include a labeled antibody for detecting leukocytes as part of a step of determining chimerism of a treated animal. The kit may also include HLA-mismatched donor stem cells, e.g., allogeneic BMT, mobilized peripheral blood cells, cord blood cells, or hematopoietic cells derived from cultured stem/progenitor cells.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent NO: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description of the Figures

Figure 1: Time course of mixed WBC chimerism in Patient 1. The percentage of donor (open bar) and host (solid bar) cells in each WBC population is shown over time. Each WBC population is normalized to 100%, so that the proportion of that particular population that is of donor vs. host origin is presented.

Figure 2: Mixed chimerism in WBC of Patient 1 one year post-BMT. Lymphocyte, monocyte and granulocyte gates are shown on the forward scatter (FSC) x side scatter (SSC) contour plot, and staining patterns with anti-HLA-A9 mAb are shown for lymphocytes (right top panel), monocytes (right middle panel) and granulocytes (right lower panel). The donor was HLA-A9-negative, whereas the host was A9-positive. The bars above the histograms denote the populations considered to be HLA-A9-negative.

Detailed Description of the Invention

(i) Overview

Bone marrow transplantation (BMT) has previously been limited from its full potential for the treatment of hematologic malignancies, due to the fact that most patients in need of an allogeneic BMT do not have HLA-matched donors available. A major obstacle to further advancement of HLA-mismatched donor BMT following standard myeloablative conditioning therapy for hematologic malignancies has been the occurrence of severe graft-versus-host disease (GVHD) and graft failure.

The present invention provides an approach which can be used in human patients in which lymphohematopoietic graft-versus-host (GVH) reactions, e.g., graft-versus-leukemia or graft-versus-lymphoma, can occur without GVHD. The non-myeloablative conditioning of the subject method permits the generation of mixed hematopoietic chimeras produced across MHC barriers, including, significantly, class II mismatches. The more potent alloresponses generated against MHC disparities compared to those against minor histocompatibility antigens usually elicits severe GVHD, which has been the major impediment to HLA-mismatched BMT (Clift et al. (1987) *Ann Rev Immunol.* 5:43-64. In certain HLA-mismatched BMT described herein, the subject protocols could not completely suppress GVHD, but in many instances it was surprisingly mild and amenable to corticosteroid therapy and the like.

(ii) Definitions

For convenience, certain terms employed in the specification, examples, and claims are collected here.

"Stromal tissue", as used herein, refers to the supporting tissue or matrix of an organ, as distinguished from its functional elements or parenchyma.

5 "Hematopoietic space", as used herein, refers to a condition created in the bone marrow which promotes engraftment of administered stem cells. In the art, hematopoietic space has often been created by irradiation of the bone marrow with whole body irradiation, but the methods of the invention generally use nonmyeloablative treatments.

10 "Hematopoietic stem cell", as used herein, refers to a cell, e.g., a bone marrow cell, or a fetal liver or spleen cell, which is capable of developing into all myeloid and lymphoid lineages and by virtue of being able to self-renew can provide long term hematopoietic reconstitution. Purified preparations of hematopoietic cells or preparations, such as bone marrow, which include other cell types, can be used in
15 methods of the invention. Although not wishing to be bound by theory, it is believed that the hematopoietic stem cells home to a site in the recipient. The preparation should include immature cells, i.e., undifferentiated hematopoietic stem cells; these desired cells can be separated out of a preparation or a complex preparation can be administered. E.g., in the case of bone marrow stem cells, the desired primitive cells can be separated
20 out of a preparation or a complex bone marrow sample including such cells can be used. Hematopoietic stem cells can be from fetal, neonatal, immature or mature animals. Stem cells derived from the cord blood of the recipient or the donor can be used in methods of the invention. See U.S. Patent 5,192,553, hereby incorporated by reference, and U.S. Patent 5,004,681, hereby incorporated by reference.

25 A "peripheral blood stem cell" is a cell with the potential to produce all the components of blood that is obtained from peripheral blood rather than from bone marrow.

An "immunosuppressive agent", as used herein, is an agent, e.g., a chemical agent, e.g., a drug, which, when administered at an appropriate dosage, results in the
30 inhibition of T cells. Examples of such agents are cyclosporine, FK-506, and rapamycin.

"Thymic or lymph node or thymocytes or T cell", as used herein, refers to thymocytes or T cells which are resistant to inactivation by traditional methods of T cell inactivation, e.g., inactivation by a single intravenous administration of anti-T cell antibodies, e.g., anti-bodies, e.g., ATG preparation.

"Thymic irradiation", as used herein, refers to a treatment in which at least 20, and preferably at least 50, 75, 90, or 95% of the administered irradiation is targeted to the thymus. Whole body irradiation, even if the thymus is irradiated in the process of delivering the whole body irradiation, is not considered thymic irradiation.

5 "MHC antigen", as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, i.e., the HLA genes.

10 The term "histocompatibility" refers to the similarity of tissue between different individuals. The level of histocompatibility describes how well matched the patient and donor are. The major histocompatibility determinants are the human leukocyte antigens (HLA). HLA typing is performed between the potential marrow donor and the potential transplant recipient to determine how close a HLA match the two are. The closer the
15 match the less the donated marrow and the patient's body will react against each other.

The term "human leukocyte antigens" or "HLA", refers to proteins (antigens) found on the surface of white blood cells and other tissues that are used to match donor and patient. For instances, a patient and potential donor may have their white blood cells tested for such HLA antigens as, HLA-A, B and DR. Each individual has two sets of
20 these antigens, one set inherited from each parent. For this reason, it is much more likely for a brother or sister to match the patient than an unrelated individual, and much more likely for persons of the same racial and ethnic backgrounds to match each other.

In hematopoietic transplantation, the word "match" relates to how similar the HLA typing is between the donor and the recipient. The best kind of match is an
25 "identical match". This means that all six of the HLA antigens (2 A antigens, 2 B antigens and 2 DR antigens) are the same between the donor and the recipient. This type of match is described as a "6 of 6" match. Donors and recipients who are "mismatched" at one antigen are considered a "5 of 6" match, and so forth.

The term "allogeneic donor stem cells" refers to cells for transplantation in a
30 subject which are derived from a family member (other than an identical twin) or from an unrelated individual, and as used herein includes cells from the same or different species, the latter being more particularly referred to as "xenogeneic".

"Hematopoietic space-creating irradiation", as used herein, refers to irradiation directed to the hematopoietic tissue, i.e., to tissue in which stem cells are found, e.g., the

bone marrow. It is of sufficient intensity to kill or inactivate a substantial number of hematopoietic cells. It is often given as whole body irradiation.

"Thymic space" as used herein, is a state created by a treatment that facilitates the migration to and/or development in the thymus of donor hematopoietic cells of a type which can delete or inactivate host thymocytes that recognize donor antigens. It is believed that the effect is mediated by elimination of host cells in the thymus.

"Tolerance", as used herein, refers to an inhibition of a graft recipient's immune response which would otherwise occur, e.g., in response to the introduction of a nonself MHC antigen into the recipient. Tolerance can involve humoral, cellular, or both humoral and cellular responses. Tolerance, as used herein, refers not only to complete immunologic tolerance to an antigen, but to partial immunologic tolerance, i.e., a degree of tolerance to an antigen which is greater than what would be seen if a method of the invention were not employed. Tolerance, as used herein, refers to a donor antigen-specific inhibition of the immune system as opposed to the broad spectrum inhibition of the immune system seen with immunosuppressants. Tolerance is the ability of the graft to survive in an MHC mismatched or xenogeneic recipient without chronic immunosuppression.

"Inhibiting immune cell activity" refers to reducing the number of active immune cells, e.g., thymocytes, T cells, B cells, or NK cells, preferably donor reactive cells, or precursor donor reactive cells, in a subject. Inhibition can include partial inhibition, or partial reduction (as opposed to total elimination) of the number of active immune cells, e.g., T cells.

The term "relapse" refers to the recurrence of illness after recovery; whereas the term "remission" refers to the disappearance of cancer cells following treatment. Also the period during which this reduction or disappearance of symptoms occur.

"Discordant species combination", as used herein, refers to two species in which hyperacute rejection occurs when a graft is grafted from one to the other. Generally, discordant species are from different orders, while non-discordant species are from the same order. For example, rats and mice are non-discordant concordant species. Concordant species combinations do not exhibit hyperacute rejection. In xenogeneic method of the invention, the donor and recipient (subject) can be a discordant or non-discordant species combination.

"Miniature swine", as used herein, refers to a miniature pig which is preferably wholly or partially inbred at at least one MHC locus. The coefficient of inbreeding of the herd which supplies the miniature swine should be at least , 0.70 and more preferably

at least 0.82. The herd from which donor animals are drawn should be homozygous at the SLA genes.

5 (iii) *Exemplary Embodiments*

Methods of the invention allow exploitation of the engraftment-promoting and GVL effects of donor T-cells while minimizing GVHD in HLA-mismatched pairs and in xenogeneic methods, allowing many more patients to benefit from hematopoietic stem cell transplantation.

10 GVL effects are mediated by T-cells and other cell types in allogeneic marrow inocula. While GVL effects are often associated with GVHD, these two phenomena can be dissociated. One strategy for separating these phenomena is the temporal separation of BMT and donor T-cell infusion. Methods of the invention provide initial conditioning, with a mild, relatively non-toxic and non-myeloablative regimen. The
15 combination of a mild conditioning regimen and the recovery time permitted before administration of donor T-cells allows the use of this approach in older patients with chronic hematologic malignancies who are otherwise not considered eligible for BMT.

Because of the high precursor frequency of T lymphocytes reacting to allogeneic MHC molecules, the anti-MHC responses in an allogeneic setting result in much more
20 potent and more rapid GVL responses than are observed for MHC-matched BMT. The presence of single HLA antigen mismatches is associated with increased GVL effects in BMT from related donors. Because of the very potency of these anti-MHC responses, and because of the ubiquity of class I MHC expression, GVHD is a major impediment to the full exploitation of this potentially enormous GVL effect. The greater
25 susceptibility of lymphohematopoietic cells than other host tissues to destruction by MHC-specific donor T-cells may be due to the immediate contact of donor cells with host cells within the lymphohematopoietic system. Additional inflammatory stimuli, such as cytokines induced by myeloablative conditioning treatments may be required to activate endothelial cells and, in combination with activation-induced increased function
30 of T-cell adhesion molecules, permit T-cell adhesion and migration into GVHD target tissues. Unfortunately, the prior art conditioning of patients for allogeneic BMT particularly harsh conditioning, may activate inflammatory stimuli, thus explaining their exquisite sensitivity to the development of GVHD. Methods of the invention avoid GVHD while preserving the strong lymphohematopoietic GVL effects of mismatched
35 allogeneic or xenogeneic donor tissue without causing GVHD, in part, by the use of

conditioning regimens that are less toxic and less pro-inflammatory, followed by delayed administration of donor T-cells. Such a delay allows recovery of host immune resistance to GVHD and/or resolution of the conditioning-induced pro-inflammatory state, and hence decreased susceptibility to GVHD. Since the host conditioning used is not myeloablative, this approach is particularly appropriate for the treatment of chronic leukemias, for which immediate curative cytoreduction need not be attempted.

Bone marrow transplantation has not been widely used for the treatment of chronic lymphocytic leukemia (CLL), an incurable and ultimately fatal disease, due largely to the fact that this disease often afflicts older patients who are not considered eligible for allogeneic bone marrow transplantation. Since CLL is a slow-growing leukemia, it is particularly amenable to cure without ablative conditioning when allogeneic T-cells are administered for their GVL effect. Additional chronic hematologic malignancies that often afflict older persons include multiple myeloma, chronic myelogenous leukemia, and low- and intermediate-grade non-Hodgkin's lymphomas are amenable to methods of the invention.

Successful allogeneic bone marrow transplantation is often limited by (1) lack of HLA-matched donor (only 25-30% of patients will have an HLA-phenotypically-identical sibling) and for patients who do undergo an allogeneic BMT, (2) substantial treatment-related mortality, particularly in patients ≥ 40 years of age, and (3) disease relapse. Methods of the invention expand the availability of transplantation by allowing transplants from HLA-mismatched donors and xenogeneic donors, improve the safety profile of BMT, and enhance the graft-versus-malignancy effects of mismatched transplantation. Methods of the invention provide a number of advantages including: (1.) A moderate dose of cyclophosphamide, a dose of 200 mg/kg, is not myeloablative and is associated with less regimen-related morbidity and mortality than conventional transplant preparative regimens. Post-chemotherapy hematopoietic recovery is expected in approximately 2 weeks following drug administration. While the decreased aggressiveness of the chemotherapy could also mean less tumor cell kill, the decreased cytoreductive effect of chemotherapy will be outweighed by an enhanced graft-versus-malignancy effect. (2.) The non-myeloablative conditioning regimen and the presumably lower likelihood of graft-versus-host disease will allow for treatment of patients of older age than are considered for conventional allogeneic BMT.

Thus, methods of the invention provide for: less toxic conditioning, which induces less host damage and less pro-inflammatory response to conditioning; partial depletion of donor T cells by administration to the subject of T cell inhibiting treatment, e.g., anti-T cell antibodies; and minimization of GVHD by delaying donor leukocyte

administration until the pro-inflammatory environment created by conditioning has receded. Methods of the invention allow the use of hematopoietic stem cells from mismatched, or xenogeneic, donors, and thus provide increased GVL activity and increases the number of individuals who can receive hematopoietic stem cell therapy for hematologic malignancies.

Methods of the invention also provide for the treatment of non-neoplastic disorders or a hemoglobinopathies, e.g., sickle cell anemia, aplastic anemia, thalassemia, or similar disorders.

In a preferred embodiment, the invention features, a method of treating a subject e.g., a human, having a hematologic disorder, e.g., a hematologic malignant disorder, e.g., leukemia.

Certain embodiments of the subject methods also feature preparative regimens which minimize or eliminate the need for myeloablative treatment, e.g., hematopoietic space-creating irradiation, especially, preparative whole body irradiation.

One aspect of the present invention provides a method for treating a subject having a hematologic disorder comprising: (i) administering a myeloreductive non-myeloablative treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (ii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form chimeric bone marrow in the subject.

In preferred embodiments each of the recited steps is a separate discrete administration or agent.

In methods described herein, the donor can be from the same species as the subject, or from a different species. In allogeneic methods the donor of stem cells and the donor of leukocytes should be the same individuals. In xenogeneic methods, the subject is a mammal, preferably a primate and more preferably a human. The donor mammal can be, by way of example, a swine, e.g., a miniature swine, or a nonhuman primate. In xenogeneic methods the donor of stem cells and the donor of leukocytes need not be the same individual but can be from different individuals which are MHC matched or highly inbred, e.g., inbred miniature swine which are MHC matched.

While not wishing to be bound by theory, the myeloreductive non-myeloablative treatment is believed to prepare the subject for the induction of mixed chimerism and may have a cytoreductive effect on cancer cells. The myeloreductive non-myeloablative treatment should be administered prior to introduction of the donor hematopoietic stem cells, preferably sufficiently prior to the administration of donor hematopoietic stem

cells such that if it includes the administration of a chemical agent, the chemical agent will be cleared from the circulatory system, e.g., preferably to a concentration of less than 0.1 of the EC₅₀ of the drug for myeloreduction, prior to the administration of donor hematopoietic stem cells.

5 In certain embodiments, the myeloreductive treatment includes treating the subject, prior to introduction of the donor stem cells, with an cytoreductive agent selected from one or more of alkylating agents (e.g., nitrogen mustards [such as mechlorethamine], cyclophosphamide, melphalan and chlorambucil), alkyl sulphonates (e.g., busulphan), nitrosoureas (e.g., carmustine, lomustine, semustine and
10 streptozocine), triazenes (e.g., dacarbazine), antimetabolites (e.g., folic acid analogs such as methotrexate), pyrimidine analogs (e.g. fluorouracil and cytarabine), purine analogs (e.g., fludarabine, idarubicin, cytosine arabinoside, mercaptopurine and thioguanine), vinca alkaloids (e.g., vinblastine, vincristine and vendesine), epipodophyllotoxins (e.g., etoposide and teniposide), antibiotics (e.g., dactinomycin, daunorubicin, doxorubicin,
15 bleomycin, plicamycin and mitomycin), dibromomannitol, deoxyspergualine, dimethyl myleran and thiotepa.

Preferred myeloreductive non-myeloablative agents are alkylating agents, e.g., cyclophosphamide, or fludarabine or similar substances, however, hematopoietic space
20 creating antibodies or drugs, e.g., inhibitors of cell proliferation, e.g., DSG, or an anti-metabolite, e.g. brequinar, or an anti-T cell antibody, e.g., one or both of an anti-CD4 or anti-CD8 antibody can be used as a myeloreductive non-myeloablative agent.

In preferred embodiments, the myeloreductive non-myeloablative treatment is sufficiently mild that at least 10, and more preferably at least 30, 50, or 75% of the
25 subjects to which it is administered will form mixed chimeras (as opposed to having their bone marrow totally ablated).

In preferred embodiments, immune cell activity, e.g., T cell activity, preferably graft reactive T cell activity, is inhibited in the subject. While not wishing to be bound
30 by theory, the inhibition of T cells is believed to prepare the subject for the induction of mixed chimerism by inhibition of subject T cell activity which would mount an immune response against the donor hematopoietic stem cells and to inhibit donor T cell activity which would mount an immune response against the subject (GVHD).

Numerous methods of inhibiting T cell activity are suitable for use in methods described herein. By way of example, these include:

the administration of anti-T cell antibodies, e.g., an ATG preparation, polyclonal
35 or monoclonal antibody directed against CD4, CD8, or CD2 (an anti-CD2 antibody, e.g.,

the anti-CD2 monoclonal antibody BTI-322 or a humanized version thereof, or an antibody which overlaps or binds the epitope recognized by BTI-322, are particularly useful);

the administration of an agent, e.g., an antibody, which blocks or otherwise
5 inhibits a pathway, e.g., a costimulatory pathway, of T cell activation (agents, e.g.,
antibodies, which block the CD28-B7 pathway, e.g., a CTLA4-IgG fusion protein, or
agents, e.g., an antibody which blocks the CD40-gp39 pathway, e.g., an anti-gp39
antibody, are particularly suited for use in the method), or generally, by the
administration of a treatment which down modulates or otherwise inhibits one or more
10 of the T cell receptor, CD4 co-receptor, CD8 co-receptor or other receptor or co-receptor
which promotes T cell activation or maturation;

the administration of an IL-12 receptor protein (functional antagonist, USSN
5,831,007);

the administration of substituted dihydrobenzofurans, spirobenzofuran-2(3H)-
15 cycloalkanes according to USSN 5,808,109;

the administration of anti-asialo antisera;

the administration of an immunosuppressive agent, e.g., a macrolide, e.g.,
cyclosporine, FK506, or rapamycin; and

the administration of thymic irradiation, or other treatment which creates thymic
20 space.

In certain embodiments, the myeloreductive treatment includes treating the
subject with an immunosuppressant regimen, prior to introduction of the donor stem
cells, in an amount sufficient to prevent rejection of the donor stem cells by the host
immune system. For example, such immunosuppressant regimens can include,
25 independently for pre- and post-transplantation is both are carried out, a treatment of the
subject which inactivates and/or depletes host T-lymphocytes and/or natural killer (NK)
cells in the subject. For example, the immunosuppressant regimen includes treatment
with T cell-depleting anti-CD4 and/or CD8 antibodies, such as anti-thymocyte globulin
(ATG), OKT3, LO-CD2a, or Minnesota anti-lymphoblast globulin (MALG).
30 Preferably, the immunosuppressant regimen, both before and after transplantation,
includes administration of ATG.

In other embodiments, the immunosuppressant regimen includes treatment with
one or more of a macrolide immunosuppressant, azathioprine, steroids (e.g., prednisone,
methyl prednisolone), or sub-lethal nonmyeloablative irradiation of lymphocyte-

containing tissue.

Treatments which inhibit T cell activity can be administered at any time in the course of the method but should not be such that donor T cells will be entirely eliminated. Treatments can be administered prior to, at the same time as, or after, the administration of donor hematopoietic stem cells. Preferably, such treatments are provided both before and after the administration of donor hematopoietic stem cells. Treatment prior to the administration of donor hematopoietic stem cells is believed desirable in that it will condition the subject for the receipt of the donor hematopoietic stem cells. Treatment after the administration of donor hematopoietic stem cells is believed desirable in that it will reduce donor-immune attack on the host and further promote acceptance by the subject of the donor hematopoietic stem cells.

For best results, treatments to inhibit T cell activity, e.g., anti-T cell antibodies or cyclosporine, can be administered repeatedly. E.g., such treatment can be administered one, two, three, or more times prior to donor bone marrow transplantation. Typically, a pre-stem cell treatment, e.g., the administration of antibodies, will be given to the patient about 1, 2, 3, 4, or 5 days prior to stem cell transplantation. It may be desirable to repeat pre-stem cell administrations every 1-5 days until the patient shows excess antibodies in the serum and about 80, 90, or 99% depletion of peripheral T cells and then to perform the stem cell transplantation. Treatments can also be administered one, two, three, or more times after donor hematopoietic stem cell transplantation. Typically, a post-stem cell transplant treatment will be given about 1, 2, 3, 4, or 5 days after bone marrow transplantation.

In preferred embodiments two or more T cell inhibiting modalities or treatments can be combined. In particularly preferred embodiments, an antibody, e.g., an anti-T cell antibody, an immunosuppressive agent, e.g., cyclosporine, and thymic irradiation, are all administered to the subject. An agent can be administered once, or more than once, but the administrations should be short term and not chronic or long term administration. In general, this will mean the treatment is administered for not more than 30, 45, 60, 90, or 120 days, and in many treatments this means administration on 1, 2, 3, 4, 5, or fewer days. Cyclosporine and similar agents will generally be administered for not more than 30, 45, 60, 90, or 120 days. Antibodies will generally be administered for 1, 2, 3, 4, 5, or fewer days.

While not wishing to be bound by theory, the donor hematopoietic stem cells are believed to provide hematologic function, and to induce tolerance to donor antigen, so as to reduce the subject response to any subsequent donor tissue, e.g., a donor leukocyte infusion, which is administered.

In preferred embodiments, mixed chimerism is induced in the subject and the state of mixed chimerism is formed in the absence of hematopoietic space created by space creating irradiation, e.g., whole body irradiation.

In preferred embodiments, donor leukocytes are administered to the subject. While not wishing to be bound by theory, the donor leukocyte administration is believed to provide additional GVL activity--donor leukocytes are believed to further and very effectively reduce the number of cancer cells in the subject. The need for or appropriateness of donor leukocyte administration can be evidenced by a lack of increase in donor chimerism, lack of GVHD symptoms, or incomplete tumor regression. Donor leukocyte administration should be delayed for at least 10, 20, 30, 35 or 60 days after the administration of any myeloreductive non-myeloablative or other space creating treatment. Initial trials showed a delay of about 35 days to be suitable. The donor leukocyte infusion is delayed to avoid introduction of relatively large numbers of donor immune cells into the host during the period in which the space creating treatment has induced pro-inflammatory conditions. Delay allows the host to recover from conditioning and to be less susceptible to GVHD, especially when mismatched donor tissue is used. The donor leukocyte infusion converts the mixed chimeric state of the subject to one which is fully chimeric, but, the graft cell mediated immune attack will be limited to the hematopoietic compartment, thereby minimizing GVHD and maximizing GVL effects.

In preferred embodiments the method includes creating thymic space in the subject. Thymic space can be created, e.g., by irradiating the thymus of the subject, e.g., by administering between 100 and 1,000, more preferably between 300 and 700, e.g., 700 rads, of thymic irradiation, or by administering anti-T cell antibodies in sufficient dose to inactivate thymocytes. Other methods for the creation of thymic space include: the administration of steroids, corticosteroids, brequinar, or an immune suppressant chemical or drug, e.g., rapamycin, cyclosporin, or FK506. An effective treatment should deplete single positive thymocytes to an extent that engraftment and the formation of mixed chimerism is optimized. In preferred embodiments the subject's single positive thymocytes are depleted by at least 20, 40, 60, or 80 %. Treatments which result in between 10 and 90 % depletion are preferred.

In preferred embodiments the subject does not receive additional treatments which stimulate the release of a cytokine by mature T cells. E.g., the subject should not receive a substance, e.g., a steroid drug, e.g., Prednisone (17, 21-dihydroxypregna-1, 4-diene-3, 11, 20-trione), at a dosage or concentration which stimulates the release of a cytokine by mature T cells in the subject. Preferably, the subject is free of such

treatment from the time stem cells are first administered until mixed chimerism is established or donor leukocytes administered.

Preferred embodiments include the administration of an agent, e.g., 15-deoxyspergualin, mycophenolate mofetil, brequinar sodium, or a similar agent, which inhibits the production, levels, or activity of antibodies in the subject.

In preferred embodiments, particularly xenogeneic methods, the method includes: inhibiting natural killer cells of the subject preferably prior to introducing donor tissue into the subject, e.g., by introducing into the subject an antibody capable of binding to natural killer cells of the subject.

One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum. A second anti-mature T cell antibody can be administered as well, which inhibits T cells as well as NK cells. Anti-T cell antibodies are present, along with anti-NK antibodies, in anti-thymocyte anti-serum. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

In preferred embodiments, the donor stem cells are provided as allogeneic bone marrow, mobilized peripheral blood cells, or cord blood cells. The donor stem cells, in some instances, can be expanded *ex vivo* for transplantation.

In preferred embodiments, particularly xenogeneic embodiments, the method includes administering donor species stromal cells or administering donor specific growth factors or cytokines, e.g., SCF or GM-SGF. Where the donor is a miniature swine, the method can include administering one or more of swine SCF, swine IL-3, or swine GM-SCF, to the subject. The method can further include the step of administering a first or subsequent dose of a cytokine or growth factor to the subject: when the subject begins to show signs of rejection; when the level of chimerism decreases; when the level of chimerism falls below a predetermined value; when the level of chimerism reaches or falls below a level where staining with a monoclonal antibody specific for a donor PBMC antigen is equal to or falls below staining with an isotype control which does not bind to PBMC's, e.g. when the donor specific monoclonal stains less than 1-2 % of the cells.

In preferred embodiments, particularly xenogeneic embodiments, the method includes the step of, preferably prior to hematopoietic stem cell transplantation, inhibiting natural subject antibodies, e.g., by depleting natural antibodies from the blood of the subject. Depletion can be achieved, by way of example, by contacting the subject's blood with an epitope which absorbs preformed anti-donor antibody. The epitope can be coupled to an insoluble substrate and provided, e.g., as an affinity

column. E.g., an α 1-3 galactose linkage epitope-affinity matrix, e.g., matrix bound linear B type VI carbohydrate, can be used to deplete natural antibodies. Depletion can also be achieved by hemoperfusing an organ, e.g., a liver or a kidney, obtained from a mammal of the donor species. (In organ hemoperfusion antibodies in the blood bind to antigens on the cell surfaces of the organ and are thus removed from the blood.) Other methods for depleting or otherwise inactivating natural antibodies can be used with the methods described herein. For example, drugs which deplete or inactivate natural antibodies, e.g., deoxyspergualin (DSG) (Bristol), or anti-IgM antibodies, can be administered to the recipient of an allograft or a xenograft. One or more of, DSG (or similar drugs), anti-IgM antibodies, and hemoperfusion, can be used to deplete or otherwise inactivate subject natural antibodies in methods of the invention.

In preferred embodiments: the donor of the hematopoietic stem cell and the donor leukocytes is the same individual. In other preferred embodiments, particularly xenogeneic embodiments, the donor of the hematopoietic stem cell and the donor leukocytes can be different individuals, e.g., different individuals which are MHC identical.

Although methods of the invention generally reduce or eliminate the need for myeloablative conditioning some embodiments include the step of, prior to hematopoietic stem cell transplantation, creating hematopoietic space for the induction of mixed chimerism by irradiating the subject with low dose, e.g., less than 400, preferably less than 300, more preferably less than 200 or 100 rads, whole body irradiation to partially deplete the bone marrow of the subject. The level of such treatment will be very substantially lower than that used in lethal conditioning. As is discussed herein, this treatment can be reduced or entirely eliminated.

The method can include a further step of treating the subject with an immunosuppressant regimen, after introduction of the donor stem cells, in an amount sufficient to prevent a graft-versus-host response mediated by the donor stem cells.

Preferably, the pre-transplantation conditioning includes administration of ATG and cyclophosphamide, and thymic irradiation. Preferably the cyclophosphamide, or other cytoreductive agents, are substantially cleared from the patient so as not inhibit proliferation of the transplanted stem cells.

An important use of the subject method is for allogeneic transplantation of donor stem cells which are mismatched, with respect to the subject, at one or more class II HLA antigens.

Another important use of the subject method is for allogeneic transplantation of

donor stem cells which are mismatched, with respect to the subject, at two or more HLA antigens (either class I or II or both).

5 In several embodiments, particularly where little, and preferably no GVHD is detected post-transplantation (e.g., at 35 days or longer), the subject method includes the further step of administering allogeneic donor leukocytes to the subject after introduction of the donor stem cells. The administration of donor leukocytes should be delayed sufficiently from the time of any hematopoietic space creating treatment such that the level of pro-inflammatory cytokines induced by the space creating treatment has subsided sufficiently to reduce or substantially eliminate GVHD from the donor
10 leukocytes.

The subject method can also include the management of GVHD responses post-transplantation by administration of immunosuppressants, or by use of engineered stem cells which give rise to small molecule ablatable T cells or other hematopoietic cells. See, for example, U.S. Patent No. 5,834,266.

15 Thus, in one preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form stable mixed chimeric bone marrow
20 in the subject.

In another preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (iii) introducing into the subject, allogeneic donor
25 hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, wherein the donor stem cells are mismatched, with respect to the patient, at one or more class II HLA antigens.

In still another preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a
30 myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, wherein the donor stem cells are mismatched, with respect to the patient, at two or more HLA antigens, e.g., class I and/or class II.

35 In yet another preferred embodiment, the subject method comprises: (i)

identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a myeloreductive treatment to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, and (iv) administering a post-transplantation immunosuppression regimen for suppressing or depleting T-cells in the transplanted donor stem cells.

In yet another preferred embodiment, the subject method comprises: (i) identifying a patient having a neoplastic hematopoietic disorder, (ii) administering a pre-transplantation conditioning to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, which pre-transplantation conditioning includes treating the cells with cyclophosphamide, ATG and thymic irradiation in an amount sufficient to reduce rejection of transplanted donor stem cells; and (iii) introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form mixed chimeric bone marrow in the subject, and (iv) administering ATG to the subject post-transplant for suppressing or depleting T-cells in the transplanted donor stem cells.

In another embodiment, the subject method includes:

administering a myeloreductive non-myeloablative treatment, e.g., an alkylating agent, e.g., cyclophosphamide, or fludarabine or a similar substance, to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, preferably without myeloablative treatment such as whole body irradiation;

preferably, inhibiting immune cell, e.g., T cell activity, in the subject;

introducing into the subject, donor hematopoietic stem cells, preferably mismatched allogeneic or xenogeneic hematopoietic stem cells, e.g., introducing donor bone marrow, to form chimeric bone marrow in the subject (as is discussed below, if a sufficiently large number of donor hematopoietic stem cells are introduced the myeloreductive non-myeloablative (hematopoietic space creating) treatment can be minimized or eliminated); and

optionally, administering to the subject, donor leukocytes, thereby treating the disorder, e.g., relieving or alleviating one or more symptoms of the disorder. The administration of donor leukocytes should be delayed sufficiently from the time of any hematopoietic space creating treatment such that the level of pro-inflammatory cytokines induced by the space creating treatment has subsided sufficiently to reduce or substantially eliminate GVHD from the donor leukocytes.

In still another embodiment the method of treating a hematologic malignancy includes the following:

administering cyclophosphamide to the subject in sufficient amount such that mixed chimerism can be induced in the subject without myeloablative treatment;

5 inhibiting T cell activity in the subject by administering thymic irradiation;

inhibiting T cell activity in the subject by administering an anti-T cell antibody, and a short course of cyclosporine both before and after the administration of donor hematopoietic stem cells;

introducing into the subject, allogeneic donor hematopoietic stem cells; and

10 optionally, administering to the subject, donor leukocytes.

In still another embodiment the method is used to treat a hemoglobinopathy, e.g., sickle cell anemia, aplastic anemia, thalassemia, or similar disorder, and includes the following:

administering a myeloreductive non-myeloablative treatment, e.g., an alkylating agent, e.g., cyclophosphamide, or fludarabine or a similar substance, to the subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, preferably without myeloablative treatment such as whole body irradiation;

preferably, inhibiting immune cell, e.g., T cell activity, in the subject; and

introducing into the subject, donor hematopoietic stem cells, e.g., introducing donor bone marrow, to form chimeric bone marrow in the subject (as is discussed below, if a sufficiently large number of donor hematopoietic stem cells are introduced the myeloreductive non-myeloablative (hematopoietic space creating) treatment can be minimized or eliminated), to thereby treat the disorder. In the treatment of non neoplastic disorders, and generally when conversion to full donor chimerism is not required, the administration of donor leukocytes can be omitted.

25 Methods of the invention can be used to treat hematologic disorders. A hematologic disorder is a disorder in which there is a malfunction in the subject's hematopoietic cells, e.g., the hematopoietic stem cells, which can be treated by replacing or supplementing the subject's hematopoietic stem cells. Hematologic disorders include disorders having unwanted cell proliferation, e.g., hematologic cancers, e.g., hematopoietic and lymphoid malignancies, e.g., leukemia, e.g., chronic lymphocytic leukemia (CLL) and other chronic hematologic malignancies, including multiple myeloma, chronic myelogenous leukemia, and low- and intermediate-grade non-Hodgkin's lymphomas. Hematologic disorders also include, non-neoplastic disorders

and hemoglobinopathies, e.g., sickle cell anemia, aplastic anemia, thalassemia, and similar disorders.

As used herein, myeloablative, refers to a treatment in which death, due to marrow failure, in a significant number of recipients, will occur if hematopoietic stem cell transplantation is not given.

As used herein, non-myeloablative, refers to a treatment which kills marrow cells but will not, in a significant number of recipients, lead to death from marrow failure.

As used herein, myeloreductive, refers to a treatment which causes cytopenia or anemia.

Subject, as used herein, refers to a mammal, e.g., a human.

Allogeneic Methods

The methods described herein can be used where, as between the donor and recipient, there is any degree of mismatch at MHC loci or other loci which influence graft rejection. Unlike conventional bone marrow transplantation, mismatch is desirable in methods of the invention, as mismatch promotes GVL effects. Methods of the invention can be used where, as between allogeneic donor and recipient, there is a mismatch at at least one MHC locus or at at least one other locus that mediates recognition and rejection, e.g., a minor antigen locus. With respect to class I and class II MHC loci, the donor and recipient can be: matched at class I and mismatched at class II; mismatched at class I and matched at class II; mismatched at class I and mismatched at class II; matched at class I, matched at class II. Mismatched, at class I or II, can mean mismatched at one or two haplotypes. Mismatched at MHC class I means mismatched for one or more MHC class I loci, e.g., in the case of humans, mismatched at one or more of HLA-A, HLA-B, or HLA-C. Mismatched at MHC class II means mismatched at one or more MHC class II loci, e.g., in the case of humans, mismatched at one or more of a DP α , a DP β , a DQ α , a DQ β , a DR α , or a DR β . In any of these combinations other loci which control recognition and rejection, e.g., minor antigen loci, can be matched or mismatched. It is preferable that there is mismatch at at least one class I or class II locus and, more preferably, mismatch at one class I and one class II locus.

The methods described herein for inducing tolerance to an allogeneic antigen or allogeneic graft can be used where, as between the donor and recipient, there is any degree of reactivity in a mixed lymphocyte assay, e.g., wherein there is no, low, intermediate, or high mixed lymphocyte reactivity between the donor and the recipient.

In preferred embodiments mixed lymphocyte reactivity is used to define mismatch for class II, and the invention includes methods for performing allogeneic grafts between individuals with any degree of mismatch at class II as defined by a mixed lymphocyte assay. Serological tests can be used to determine mismatch at class I or II loci and the invention includes methods for performing allogeneic grafts between individuals with any degree of mismatch at class I and or II as measured with serological methods. In a preferred embodiment, the invention features methods for performing allogeneic grafts between individuals which, as determined by serological and or mixed lymphocyte reactivity assay, are mismatched at both class I and class II.

10 In preferred embodiments the donor and the subject are not related, e.g., the donor is not a sibling, the offspring of, or the parent of the recipient.

Xenogeneic Methods

15 Methods of the invention can use xenogeneic donors. E.g., when the subject is a human, the donor can be a non-human primate or a swine, preferably a miniature swine.

Hematopoietic Stem Cells

20 Methods of the invention require the introduction of donor hematopoietic stem cells. Administration and engraftment of the donor stem cells converts the subject to a mixed chimera. Because donor hematopoietic stem cells are at a competitive disadvantage to subject hematopoietic stem cells, it is often desirable to create hematopoietic space in the donor, in order to promote engraftment of the donor cells. Methods of the invention use mild non-myeloablative methods, e.g., the administration of cyclophosphamide, to create hematopoietic space. However, if a sufficient number of donor cells are administered, the subject need not receive space-creating treatment. See e.g., US patent application 08/855,705, filed May 8, 1997, hereby incorporated by reference. Thus, other methods of the invention administer a sufficient number of donor hematopoietic stem cells such that the creation of space, even with mild methods, is not required. This approach is particularly useful in xenogeneic methods, especially those in which very large numbers of donor hematopoietic stem cells are available, e.g., when the donor or donors are inbred miniature swine.

30 The number of donor stem cells administered to the recipient can be increased by either increasing the number of stem cells provided in a particular administration or by providing repeated administrations of donor stem cells.

Repeated stem cell administration can promote engraftment and mixed chimerism in recipients. In preferred embodiments, particularly xenogeneic embodiments, multiple administrations of donor stem cells can be provided. A second (or other subsequent) administration of hematopoietic stem cell can be provided: at least
5 two days, one week, one month, or six months after the previous administration of stem cells; when tumor regression is below desired levels; when the level of chimerism decreases; when the level of chimerism falls below a predetermined value; when the level of chimerism reaches or falls below a level where staining with a monoclonal antibody specific for a donor PBMC antigen is equal to or falls below staining with an
10 isotype control which does not bind to PBMC's, e.g. when the donor specific monoclonal stains less than 1-2 % of the cells; or generally, as is needed to maintain tumor regression.

When multiple stem cell administrations are given one or more of the administrations can include a number of donor hematopoietic cells which is at least
15 twice, is equal to, or is at least 75, 50, or 25% as great as, the number of bone marrow cells found in an adult of the recipient species; include a number of donor hematopoietic stem cells which is at least twice, is equal to, or is at least 75, 50, or 25% as great as, the number of bone marrow hematopoietic stem cells found in an adult of the recipient species. Such large numbers are useful in reducing or eliminating the need for space
20 creating treatment, even mild treatments.

The method of introducing stem cells may be altered, particularly by (1) increasing the time interval between administering hematopoietic stem cells and space creating treatment or leukocyte infusion; (2) increasing the amount of hematopoietic stem cells injected; (3) varying the number of hematopoietic stem cell injections; (4)
25 varying the method of delivery of hematopoietic stem cells; (5) varying the tissue source of hematopoietic stem cells, e.g., a fetal liver cell suspension may be used; or (6) varying the donor source of hematopoietic stem cells. Although hematopoietic stem cells derived from the leukocyte donor are preferable, hematopoietic stem cells may be obtained from other individuals or species, or from genetically-engineered inbred donor
30 strains, or from *in vitro* cell culture.

Sources of Cells for Allogeneic Stem Cell Transplantation

A living human donor can provide about 7.5×10^8 bone marrow cells/kg. Methods of the invention can include the administration of at least 2 or 3 times this
35 number (per kg) especially when it is desired to reduce or eliminate space creating

treatments, and preferably at least 10, 15, or 20 times this number. Such large numbers are useful in reducing or eliminating the need for space creating treatment, even mild treatments. The requisite numbers of bone marrow cells can be provided by the ex vivo expansion or amplification of human stem cells. Ex vivo expansion is reviewed in Emerson, 1996, Blood 87:3082, hereby incorporated by reference. Methods of ex vivo expansion are described in more detail in Petzer et al., 1996, Proc. Natl. Acad. Sci. USA 93:1470; Zundstra et al., 1994, BioTechnology 12:909; and WO 95 11692 Davis et al., all of which are hereby incorporated by reference. Sources of hematopoietic stem cells include bone marrow cells, mobilized peripheral blood cells, and when available cord blood cells.

The hematopoietic system reconstituting cells administered to the recipient can, in one example, be present in a source population of between 0.2×10^8 and 4.0×10^8 , or ranges there between, donor bone marrow cells/kg of the recipient's body weight. The bone marrow cells can be obtained from the donor by standard bone marrow aspiration techniques known in the art. Bone marrow cells are removed from the donor by placing a hollow needle into the marrow space and withdrawing a quantity of marrow cells by aspiration.

Alternatively, the hematopoietic system reconstituting cells administered to the recipient can, in one example, be present in a source population of between 1.0×10^8 and 40×10^8 , or ranges there between, donor cytokine mobilized peripheral blood stem cells/kg of recipient's body weight. Peripheral blood cells can be obtained from the donor, for example, by standard phlebotomy or apheresis techniques. Phlebotomy is performed by placing a hollow needle into a vein and withdrawing a quantity of whole blood using aspiration or gravity. Apheresis is performed in a similar manner to phlebotomy except the whole blood is anticoagulated and then separated into the constituent formed cellular elements by centrifugation. The mononuclear cell fraction is retained and the remaining plasma and other cellular elements (red blood cells, granulocytes, platelets) are returned to the donor by intravenous infusion.

Peripheral blood stem cells can be cytokine mobilized by injecting the donor with hematopoietic growth factors such as Granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), stem cell factor (SCF) subcutaneously or intravenously in amounts sufficient to cause movement of hematopoietic stem cells from the bone marrow space into the peripheral circulation. The hematopoietic reconstituting cells can also be derived from fetal or embryonic human tissue that is processed and/or cultured in vitro so as to increase the numbers or purity of primitive hematopoietic elements.

In addition, the hematopoietic system reconstituting cells administered to the recipient can also be hematopoietic system cells that have been enriched from the source population. The source population can be either donor bone marrow cells or donor peripheral blood cells. The hematopoietic system reconstituting cells can be enriched from the source population by selecting cells that express the CD34 antigen, using combinations of density centrifugation, immuno-magnetic bead purification, affinity chromatography, and fluorescent activated cell sorting, known to those skilled in the art (Baum et al., (1992) Proc. Natl. Acad. Sci. U.S.A. 89:2804-8; Lansdorp et al., (1990) J Exp. Med. 172:363-6; Sato et al., (1991) Blood 78:967-74; Smith et al., (1991) Blood 77:2122-8; Udomsakdi et al., (1991) Exp. Hematol 19:338-42; Udomsakdi et al., (1992) Blood 80:2513-21.

The treated mononuclear cells and hematopoietic system reconstituting cells are typically administered to the recipient in a pharmaceutically acceptable carrier by intravenous infusion. Carriers for these cells can include but are not limited to solutions of phosphate buffered saline (PBS) containing a mixture of salts in physiologic concentrations.

Sources of Cells for Xenogeneic Stem Cell Transplantation

In the case of inbred donor animals, e.g., inbred miniature swine, very large numbers of stem cells are available, as the number which can be supplied is not limited by the number which can be harvested from a single donor.

In the case where the recipient is a primate, e.g., a human, and the donor is a swine, e.g., a miniature swine, 7.5×10^9 or more, and preferably, between 7.5×10^9 and 15×10^{10} , swine bone marrow cells/kg can be administered, though this will vary with factors such as the intensity of the preparative regimen and the health of the individual recipient. Such large numbers are useful in reducing or eliminating the need for space creating treatment, even mild treatments. As discussed herein, these cells can be provided in more than one administrations.

Treatment of Hematologic Cancers

The following provides a protocol for the treatment of a human subject having a hematologic cancer in a preliminary clinical setting. The protocol describes the major components of the treatment, the therapy to be provided to the patient, pre- and post-treatment evaluation, and supportive care likely to be needed in the course of the

treatment. This protocol is exemplary of an embodiment of the invention and is not limiting.

The treatment consists of four major components:

1. Conditioning therapy, e.g., with cyclophosphamide 200 mg/kg and thymic irradiation (7 Gy) and BMT.
2. GVHD prophylaxis, e.g., with anti-thymocyte globulin (ATG) and cyclosporine.
3. Post-transplant supportive care (antibiotics, transfusional support, hemopoietic growth factors, etc.)
4. Donor leukocyte infusions (days +35, +56).
5. Thymic irradiation except patients who have received previous mediastinal radiation therapy.

Scheme of therapy is as follows:

Day	Treatment
-6	Cyclophosphamide 50 mg/kg
-5	Cyclophosphamide 50 mg/kg
-4	Cyclophosphamide 50 mg/kg
-3	Cyclophosphamide 50 mg/kg
-2	ATG 15 mg/kg
-1	Thymic irradiation (7 Gy) ATG 15 mg/kg CYA 5 mg/kg IV
0	Bone marrow infusion CYA 5 mg/kg IV
+1	ATG 15 mg/kg
+4	CYA 3 mg/kg IV
+15	CYA 12 mg/kg PO
+30	CYA 12 mg/kg PO
+35	Donor leukocyte infusion
+56	Donor leukocyte infusion

Treatment modalities referred to in the scheme of therapy are as follows:

A. Cyclophosphamide (Cytosan™)

1. Dosage: cyclophosphamide is administered at a dosage of 50 mg/kg on days -6, -5, -4, and -3. cyclophosphamide is dissolved in distilled water and administered over 60 minutes. Dose should be calculated based on actual or ideal body weight, whichever is less. Volume of distilled water to be used is 250 ml for adults.
2. Sedation, antinausea: Dexamethasone, Diphenhydramine, Lorazepam and Granisetron prior to cyclophosphamide.
3. Because of a 20% incidence of hemorrhagic cystitis, the following plan of fluid administration and management is recommended for prevention:
 - a. IV hydration fluids for adults should be at 3000 ml/m²/24 hours, beginning 4 hours prior to cyclophosphamide administration. Typically the hydration fluid is D₅NS + 20 mEq KCL/liter. This fluid should be continued for 24 hours after the last dose of cyclophosphamide.
 - b. MESNA at a dose of 15 mg/kg will be administered 15 minutes before and 3, 6, and 9 hours after cyclophosphamide (with an additional dose 24 hours after the fourth IV dose).
 - c. Additional KCL and NaHCO₂ may be needed depending on patient's electrolyte and uric acid status.
4. Toxicity and complications
 - a. Nausea and vomiting. Variable but usually well controlled with anti emetics.
 - b. Uric acid nephropathy. A potential problem that is easily prevented by high urine flow plus alkalinization and allopurinol.
 - c. Fluid retention. cyclophosphamide causes an antidiuretic effect usually counteracted by furosemide administration. Careful physical examination and accurate weights three times a day should be able to detect fluid overload early.

- 5 d. Cardiomyopathy. cyclophosphamide causes nonspecific ST changes at this dose level and at total doses ≥ 200 mg/kg (7.6 grams/m²) fatal cardiac failure due to hemorrhagic necrosis can occur. cyclophosphamide is contraindicated in patients with pre-existing cardiac disease. Patients should receive an EKG on admission, on each day cyclophosphamide is given, and 1 day following cyclophosphamide.
- 10 e. Diarrhea. May be a problem and should be treated symptomatically with Tincture of Opium (dose is 1-3 drops/dose) or Immodium. Stool volume loss should be replaced with D₅W and an appropriate electrolyte solution.
- 15 f. Hemorrhagic cystitis. Approximately 50% of patients will have some hematuria at this dose level, but is usually not symptomatic or severe unless there is inadequate diuresis. An occasional patient will get severe cystitis despite adequate urine flow.
- g. Alopecia. The patient should be told of hair loss prior to drug administration.
- 20 h. Skin rash. 10-20% of patients develop a diffuse maculopapular rash 24-72 hours following cyclophosphamide. The rash usually resolves in 24-48 hours.
- i. Anemia. Hematocrit decrements out of proportion to cessation of production will occur at this dose, presumably due to hemolysis.
- 25 j. Electrolyte imbalance. This should be anticipated and daily electrolytes followed.

B. Anti-thymocyte Globulin (ATG; ATGAM™, Upjohn Co.)

- 30 1. Dosage: ATG is prepared in 1 liter of normal saline and is given at a dose of 15 mg/kg over 10-12 hours on days -2, -1, and +1. The dose of ATG will be based on ideal or actual body weight, whichever is less.
2. Skin Testing

- 5 a. All patients will receive an intradermal skin test (0.1 ml of a 1 mg/ml solution) and observed for 30 minutes for the presence of a wheal/flare reaction. If positive, an alternative treatment plan may be considered by the principal investigator. Benadryl 50 mg IV, epinephrine (1:1000 solution) and hydrocortisone 100 mg IV will be available at the bedside in the event of a possible allergic reaction.

3. Pre-Medication

- 10 a. All patients will receive dexamethasone 10 mg IV Q 12 hrs on days -2, -1, and +1. Each ATG infusion will be preceded by Benadryl IV and Tylenol 650 mg.

4. Toxicities

- 15 a. Allergic reactions (including anaphylaxis), rash, fevers, rigors, arthralgias, myalgias, dyspnea, serum sickness (including rash, arthritis, proteinuria), hypotension, tachycardia.

C. Cyclosporine (Sandimmune™, Sandoz Co.)

- 20 1. Cyclosporine is commercially available and is administered either in an intravenous form (mixed in 250 ml of distilled water), or an oral olive oil based solution, or in capsule form (100 and 25 mg capsules).

- 25 2. All patients will receive cyclosporine starting on day -1 at a dose of 5 mg/kg/day intravenously infused over a period of 20 hours daily. The dose will be reduced to 3 mg/kg/day on day +4 until the patient is able to tolerate p.o. cyclosporine (on or after day +15 post-transplant) at a dose of 6 mg/kg twice daily. Cyclosporine will be dose adjusted on the following criteria:

- 30 a. Cyclosporine levels: an attempt will be made to keep cyclosporine dose levels within the therapeutic range (between 250-350 mg/ml by monoclonal assay). Given an association between low cyclosporine levels and the development of acute GVHD, attempts will be made to keep

the level in the high normal range particularly during the first 4 weeks post transplant.

- 5 b. Dose reduction should be considered for significant renal dysfunction (e.g. greater than a 50% increase from baseline serum creatinine level particularly accompanied by a high cyclosporine level).
- c. Careful attention should be given to cyclosporine levels and renal function in the face of hepatic disease, given the extent of hepatic metabolism of cyclosporine.
- 10 d. In the absence of acute GVHD, cyclosporine will be tapered and discontinued by day +30 post-transplant.

D. Thymic Irradiation

- 15 1. 7 Gy of thymic irradiation will be administered in a single dose on day -1.
2. Possible toxicities of thymic irradiation include bone marrow suppression, nausea, vomiting, esophagitis, pneumonitis, pericarditis and secondary malignancy.

20 E. Bone Marrow Infusion

1. Allogeneic bone marrow will be rapidly infused intravenously without a filter as soon as possible after harvest.
- a. Acute toxicities:
- 25 1. Pulmonary emboli. Marrow and fat emboli may rarely cause a transient alveolar capillary block and temporary administration of O₂ may be necessary.
2. Hypotension.
3. Volume overload.

30 F. Donor Leukocyte Infusion(s)

- 5
1. Donor peripheral blood mononuclear cells will be collected via leukapheresis on days +35 and +56 post-transplant. Based on established anti-tumor efficacy of $1 \times 10^7/\text{kg}$ T-cells in CML and reduced risk of GVHD with this dose compared with $\geq 5 \times 10^7/\text{kg}$ T-cells (34), an initial infusion (day +35) of $1 \times 10^7/\text{kg}$ CD3 + T-cells will be performed. If no GVHD is observed and fully donor (greater than or equal to 90%) chimerism has not been established, or if there is evidence of persistent malignancy, a second dose of $10^7/\text{kg}$ T-cells will be infused on day +56 post-transplant.
 - 10 2. Recipient risks of receiving donor leukocyte infusions include acute and chronic GVHD and marrow aplasia.

Evaluation

15 The following protocol can be used to evaluate prospective recipients.

A. Pre-transplant

1. History. A complete history with full details of the patient's previous treatment and response will be obtained, including:

20 a. Patient exposure to steroids, radiation and antileukemic drugs (total dosage of each antileukemic drug and when given).

b. Previous or current fever, infections and antibiotic treatment.

c. Previous CNS involvement and other evidence of extramedullary leukemia.

25 d. Clinical picture at initial presentation including Kamofsky score.

e. Prior immunologic and cytogenetic studies of the patient's leukemic cells.
- 30 2. Clinical evaluation (all measurements in metric units).
 - a. A complete physical examination.

- 5
- b. Chest and other radiographs as clinically indicated.
 - c. Marrow aspiration and biopsy for staging and cytogenetics.
 - d. EKG
 - e. Dental consult and evaluation of status of teeth and gums.
 - f. Lumbar puncture(s) for determination of presence of CNS leukemia and administration of IT therapy for patients with intermediate to high grade non-Hodgkin's lymphoma.
 - g. Pulmonary consult for baseline respiratory studies, with room air arterial blood gas.
- 10
3. The following laboratory data should be obtained:
- a. ABO and Rh typing and two-way red cell crossmatch with donor.
 - b. HLA typing of patient and available family members and potential platelet donors.
 - c. Hepatitis B surface antigen, HCV, HSV, CMV, HIV and HTLV-1 antibody determinations for patient and marrow donors.
 - d. Cultures of blood, stool, urine, nose, and throat for potential pathogenic bacteria, viruses and fungi.
 - e. CBC, reticulocyte count, chem 20 and toxoplasma titers. 10 ml serum (2 dry red tops) and 20 cc of heparinized blood (2 green tops) for immunologic studies to Dr. Spitzer.
 - f. Lymphoma or leukemia cells, from marrow or peripheral blood, if available, to be frozen in DMSO for later immunologic studies (send to Dr. Spitzer).
- 15
- 20
- 25

The following protocol can be used to monitor patients who receive treatment with methods of the invention.

30

B. Evaluation during conditioning and the first 100 days post-transplantation

1. Daily CBC until granulocytes and platelets are self-sustaining; at least three times weekly until discharge, and then once or twice weekly until 100 days post-transplant.
2. Daily chemistry profile for the first 3 weeks, then as clinically indicated, but at least once weekly.
3. Marrow aspiration and biopsy on day 28 and 100 post-transplant.
4. Chest x-ray every 7 days.
5. Serum to serum bank (10 ml clotted blood to Dr. Spitzer) every 7 days.
6. Viral, bacterial and fungal cultures weekly or when clinically indicated or as specified in other protocols until discharge, then as clinically indicated.
7. EKG daily during and 1 day after last dose of cyclophosphamide.
8. Daily weights.
9. Respiratory function tests per attending.
10. Blood for CSP levels every Monday or as per attending (7 mg lavender EDTA tube).
11. Screening studies for chronic GVHD on day 100 (per Dr. Spitzer).
12. Chimerism studies: see study parameter (section XI).

C. Evaluation following 100 days post-transplant

1. Monthly evaluations here for local patients and by referring physicians for patients who live elsewhere for one year following engraftment.
2. Monthly CBC and chem 20 for 6 months. Marrow aspiration as indicated clinically or required by other protocols.
3. Periodic studies as per other specific protocols.
4. Complete evaluation every 6 months for 2 years, then annually.

Supportive Care

The following outlines supportive care which may be appropriate.

- 5 A. Access to vessels. All patients will have placement of a silastic indwelling triple lumen central venous catheter (or have dual double lumen catheters) on or before admission.
- 10 B. Hyperalimentation (HAL). Some patients will require hyperalimentation soon after conditioning. Given the hepatotoxic potential of HAL, caloric intake will be adjusted to provide approximately 50 to 75% of the calculated need.
- 15 C. Transfusions.
1. Indication. Platelets are transfused to prevent bleeding and an attempt will be made to keep the circulating platelet level $> 20 \times 10^9/L$ at all times. Packed red blood cells will be transfused to maintain a hematocrit of $\geq 25\%$.
- 20 2. Single donor apheresis platelet products containing a minimum equivalent of 6 units of random donor platelets will be preferentially used.
3. Patients will have their CMV serostatus determined prior to conditioning therapy. CMV negative patients will receive CMV negative blood products as available.
- 25 4. All platelet and red cell transfusions will be white blood depleted using third generation leukocyte filters.
5. Irradiation. All products are irradiated with 2500 rad (from a ^{137}Cs irradiator) prior to infusion to inactivate lymphocytes and prevent a transfusion associated GVHD.
- 30 6. Problems.
- a. Alloimmunization. Patients frequently become refractory to mismatched platelets due to alloimmunization to HLA and platelet antigens. For practical purposes, this is established

5 by failure to demonstrate an adequate increment 60 minutes after a 4-6 unit equivalent transfusion. If non-family platelets are required and the patient is immunized to random donors, attempts should be made to locate an unrelated platelet donor with no HLA antigens in excess of the recipient. Mismatched platelets may be harmful in the presence of alloimmunization, with chills, fever and a drop in circulating platelet and neutrophil levels.

10 b. Consumption. These patients have complicated problems leading to rapid consumption of platelets, and the distinction between this and alloimmunization is often difficult. Patients who demonstrate an adequate post-transfusion increment but have rapid disappearance of platelets are assumed to have consumption and will be given more frequent platelet transfusions.

15 c. Allergic reaction. Chills, fever, and hives occasionally occur despite adequate circulation of platelets. These are presumed to be allergic reactions to antigens other than HLA or platelet antigens and can be controlled with diphenhydramine. However, if associated with no increment or with a decrement, these reactions are probably associated with alloimmunization to the transfused platelets and that donor should not be used again.

25 D. Management of infections. Principles of infection prophylaxis and treatment will vary according to the spectrum of organisms and their antibiotic sensitivity and concurrent infection management/antibiotic clinical trials. General principles of infection management will include:

- 30
1. reduced bacteria diet
 2. HEPA filtered or LAF protective isolation
 3. Oral Ofloxacin 400 mg BID, from admission until ANC is $>0.5 \times 10^9/L$ for antibacterial prophylaxis.
 4. Acyclovir 250 mg/m² IV or PO q8h days -1 until discharge for HSV prophylaxis.

5. Fluconazole PO or IV days -1 until ANC is $>0.5 \times 10^9/L$ for antifungal prophylaxis.
6. Broad spectrum antibiotics for fever ($T \geq 100.5$) in the face of neutropenia with continuation of antibiotics until ANC is $>0.5 \times 10^9/L$. Antibiotic choice will vary but will usually consist of vancomycin and a third generation cephalosporin (e.g. ceftazidime) or imipenem. Aminoglycosides should be avoided if possible in view of potential synergistic renal toxicity with CSP and IL2.
7. For CMV prophylaxis/therapy:
- CMV negative blood products for CMV seronegative recipients.
 - IVIG 500 mg/kg/week days -8 through +28, then every other week through day +100.
 - DHPG for positive CMV culture or positive antigen assay.

These procedures may be employed in combination, as described, or in part. These procedures are designed to synergistically prevent the problem of GVHD while maximizing the GVL effect of donor tissue.

(iv) Exemplification

The invention now being generally described will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1

Mixed lymphohematopoietic chimerism following a non-myeloablative conditioning regimen and allogeneic bone marrow transplant (BMT)

Five patients with chemotherapy (n=5) and radiation (n=2) refractory non-Hodgkin's lymphoma were treated with cyclophosphamide (Cy) 50 mg/kg daily x 4 (days -6 through -3), anti-thymocyte globulin 30 mg/kg (days -2, -1, +1) thymic irradiation 700 cGy x 1 (n=3) (day -1) and HLA genotypically identical (n=2),

phenotypically identical (n=1), or 2 antigen mismatched donor BMT (day 0). Intravenous cyclosporine (CYA) was given beginning on day -1 with conversion to p.o. CYA when tolerated. Donor leukocytes (DLI) were given on days +35, ($10^7/\text{kg}$ CD3+ cells) and +56 ($5 \times 10^7/\text{kg}$ CD3+ cells) if no GVHD was present. Median patient age was 30 (range 20-45) years. All patients had disease progression during salvage chemotherapy or radiation therapy. Toxicities have included reversible Cy cardiotoxicity (n=1), and capillary leak syndrome coincident with engraftment (n=4). Median times to ANC > 0.5 and platelets > 20K have been 16 (range 13-17) and 16 (range 8 to 91 +) days. Chimerism analyses of weekly peripheral blood samples and pre-BMT, day +28, and day +100 bone marrow aspirate samples have been performed by variable number of tandem repeat sequence (VNTR) analysis (HLA matched BMT) or flow cytometry (HLA mismatched BMT). In recipients of HLA-matched marrow (n=3) mixed chimerism was seen in all three. One patient had conversion to full donor chimerism following DLI and developed grade III GI GVHD. One patient had $\leq 10\%$ donor cells; at day +35 he had no detectable donor cells. No donor cells were detectable after a subsequent DLI. The third patient has 50-70% donor cells on day +28 with grade II cutaneous GVHD. In the two recipients of HLA-2 antigen mismatched marrow, >90% donor lymphoid chimerism was seen within 2 weeks of BMT coincident with grade II or III GVHD. In one patient gradual conversion to donor myeloid (neutrophils and monocytes) chimerism was observed. In the other patient stable "split" lymphohematopoietic chimerism is present (>90% donor lymphoid, >80% host myeloid). All patients are alive at a median of 103 days (range 3y-122 days) following BMT. Four of five patients are clinically disease-free at the present time. Mixed lymphohematopoietic chimerism is achievable following a novel non-myeloablative conditioning regimen and HLA-matched or mismatched BMT. Dramatic anti-tumor responses have been seen in the majority of cases.

Example 2

Activation-induced cell death in donor TCR transgenic T cells with known host antigen specificity

The expansion and elimination of donor T cell receptor (TCR) transgenic T cells with known host antigen specificity was examined in a murine BMT model. In 2C T-cell receptor transgenic mice (H-2^b on B6 background), a large fraction of T cells express CD8 and the $\alpha\beta$ T-cell receptor from the cytotoxic T lymphocyte clone 2C, which specifically recognizes the MHC class I antigen L^d (Sha, W.C. et al., Nature 335, 271-274 1988). Lethally irradiated, L^d+ BALB/c (H-2^d) mice were transplanted with

10x10⁶ spleen cells from anti-L^d 2C TCR-transgenic B6 mice. By 4 days after BMT, the numbers of 2C CD8 cells in the spleens of BALB/c recipients had increased 14-16 fold over the number administered. However, they had drastically decreased to similar numbers to those administered by day 7. By 3-color FCM using 7-AAD (amino-actinomycin-D) for DNA staining, we found that an increasing fraction (4-11%) of
5 GVH-reactive 2C CD8 cells in spleens of 2C→BALB/c recipients underwent apoptotic cell death between days 4 and 7 after BMT, coincident with the observed decrease in absolute numbers of 2C CD8 cells in recipients' spleens. In addition, 2C CD8 cells showed reduced expression of 2C TCR and CD8 on days 4, 7, and 21. They also
10 demonstrated anergy upon stimulation with anti-αβTCR and 1B2 (anti-2C clonotypic mAb) mAbs at these time points. 2C CD8 cells remaining on day +21 expressed the CD45RB^{low} CD44^{high} Mel14^{low}, previously activated/memory phenotype. Clinically, the recipients did not show any evidence of acute GVHD, and all animals survived beyond day 80, although some of them exhibited mild chronic GVHD. The early clonal
15 expansion of 2C CD8 cells, downregulation of CD8 and TCR, anergy and deletion of 2C CD8 cells via activation-induced cell death, are all the consequences of a vigorous immune response. However, this marked monoclonal expansion of GVH-reactive 2C CD8 cells is incapable of inducing severe acute or subacute GVHD.

20 **Example 3**
Mixed Lymphohematopoietic Chimerism and Graft-vs-Lymphoma Effects Are Achievable in Adult Recipients Following Non-Myeloablative Therapy and HLA-Mismatched Donor Bone Marrow Transplantation

25 *Methods:* Five patients with refractory non-Hodgkins lymphomas underwent BMT from two of six HLA antigen-mismatched (in the GVH direction) donors. Conditioning included pre-transplant cyclophosphamide, pre- and post-transplant anti-thymocyte globulin (ATG), and pre-transplant thymic irradiation. Additional GVHD prophylaxis consisted only of cyclosporin A.

30 *Findings:* Four of four evaluable patients engrafted, and mixed hematopoietic chimerism was established, with donor lymphoid predominance and varying levels of myeloid chimerism. Two patients are in GVHD-free complete and partial clinical remissions at 460 and 103 days post-BMT, respectively.

35 *Interpretation:* This is the first demonstration that mixed chimerism can be intentionally induced in adult recipients of HLA-mismatched BMT. Moreover, this has been achieved using a non-myeloablative conditioning regimen. The striking anti-

lymphoma responses seen in several patients suggest that allogeneic BMT can have potent immunotherapeutic benefits in the absence of myeloablative conditioning.

Introduction

5 Patients with chemo- and radio-resistant non-Hodgkins lymphomas (NHL) have a very poor prognosis. HLA-identical allogeneic or autologous bone marrow transplantation has led to durable remissions in only 0-23% of patients ^{1,2}. However, animal studies have shown that MHC-disparate bone marrow transplants can mediate anti-tumor effects that greatly exceed those achieved with MHC-matched BMT ^{3,4}. The potential of HLA-mismatched bone marrow transplantation as immunotherapy for 10 hematologic malignancies has not yet been exploited, largely because of the high incidence of intractable GVHD ⁵ and of potentially lethal failure of marrow engraftment associated with standard ablative conditioning regimens ⁶⁻⁸.

Studies in rodents have shown that mixed hematopoietic chimeras produced 15 across MHC barriers are resistant to the development of GVHD, even when lymphohematopoietic GVH reactions are intentionally induced that convert mixed chimeras to fully allogeneic chimeras ⁹ (M.-G. Wang and M. Sykes, unpublished data). Murine mixed chimeras produced with a non-myeloablative conditioning regimen of T cell-depleting mAbs, cyclophosphamide (CP), and thymic irradiation (TI), can be 20 converted into full donor chimeras without developing GVHD when donor lymphocytes are administered 5 weeks post-BMT. We have now adapted the mixed chimerism approach for use in humans with hematologic malignancies, using CP for both cytoreduction of malignancy and as an adjunct to host immunosuppression with anti-thymocyte globulin and TI. We show here that cytoreductive, immunosuppressive, but 25 non-myeloablative conditioning administered to patients with refractory hematologic malignancies can induce stable mixed chimerism with potent graft-versus-lymphoma effects.

Patients and methods

30 *Patients*

The five patients described herein were enrolled at the Massachusetts General Hospital in a trial involving non-myeloablative conditioning therapy followed by allogeneic BMT, under the auspices of an MGH Subcommittee for Human Studies-approved protocol. Eligibility criteria included chemotherapy-refractory hematologic

malignancy, ECOG performance status of 2 or less, age of 65 years or less and adequate organ function (as specified by the protocol). A less than three of six HLA antigen-mismatched related donor was required. Patients and donors were typed using standard serological techniques for HLA-A and B, and SSOP-or SSP-based analyses for HLA-DR.

The characteristics of the HLA-mismatched allogeneic transplant recipients are listed in Table 1. All five had intermediate- to high-grade non-Hodgkins lymphomas that were refractory to chemotherapy \pm radiotherapy, and received transplants from two of six (A, B, or DR) HLA antigen-mismatched (in the GVH direction) donors.

10

Conditioning and transplantation

Conditioning therapy consisted of intravenous cyclophosphamide (CP) 50 mg/kg/d (with dosing based on actual or ideal body weight, whichever was less) on days -6 through -3, thymic irradiation (700 cGy) on day -1 in patients who had not received previous mediastinal radiation therapy (n=2), and anti-thymocyte globulin (ATG) 30 mg/kg/d (n=2) or 15 mg/kg/d (n=2) on days -2, -1, and +1 (Patients 1 through 4), or 15 mg/kg/d on days -1, +1, +3, +5 (Patient 5). Dexamethasone was used at a dose of 20 mg/d prior to each dose of CP and at a dose of 10 mg twice daily with each dose of ATG. Intravenous cyclosporine, 5 mg/kg daily, was given on day -1 until day +4, when it was reduced to 3 mg/kg/d. When oral medication was tolerated, cyclosporine was changed to an oral route at a dose of 6 mg/kg q12h.

Donor bone marrow was procured under anesthesia by standard techniques. A target number of 3×10^8 /kg nucleated cells was sought. In the case of minor ABO incompatibility (n=1), plasma was removed from donor marrow prior to transplantation. In the case of major ABO-incompatibility (n=1), red blood cells were depleted from the donor marrow using a CS-3000 cell separator (Baxter-Fenwal, Round Lake, IL).

Analyses of chimerism

Flow cytometry (FCM) was used for analysis of white blood cells stained with FITC-labeled anti-HLA class I allele-specific mAbs (One Lambda, Inc.; Canoga Park, CA) specific for the HLA type of the patient (HLA-Bw4 or A9) or the donor (HLA-A3). These mAbs were used in combination with PE-, PerCP- or APC-conjugated antibodies to the human lymphocyte differentiation antigens CD3, CD4, CD8, CD19, and CD56

(Becton Dickinson). Staining, flow cytometer calibration and analysis was performed with standard techniques ¹⁰ on approximately 50,000 cells per analysis tube.

In four of the five recipients, analysis of minisatellite variable number of tandem repeats (VNTR) or short tandem repeat (STR) markers ^{11,12} were also able to distinguish
5 donor and host. Donor or recipient bands were detectable in mixtures containing as little as 1% of the DNA from the donor or recipient, respectively.

Erythroid chimerism was determined in Patient 1 (recipient blood group A, donor O) by the gel test method of Lapierre ¹³, using Micro Typing Systems (MTS) (Pompano Beach, FL) buffered gel cards with anti-A mAb and anti-H lectin to agglutinate type A
10 and type O RBC, respectively. The sum of A⁺ and H⁺ RBC totaled approximately 100%, indicating that all RBC typed as being of either donor or host origin.

RESULTS

Clinical outcomes

15 The clinical courses of the five HLA-mismatched patients in our trial are summarized in Table 2. The patient with the longest follow-up (Patient 1) is described in detail. This 20-year-old male presented with a left neck mass in May 1996, followed two months later by rigors and night sweats. Biopsy of the mass established the diagnosis of non-Hodgkins lymphoma, diffuse large cell type. Extensive cervical and
20 mediastinal lymphadenopathy with pulmonary involvement and a pericardial effusion were present, indicating stage IVB disease. An initial partial response to cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy was followed by progression of cervical lymphadenopathy during the sixth cycle. The patient's cervical disease progressed through subsequent chemotherapy with etoposide,
25 cisplatin, cytosine arabinoside, and methyl-prednisolone (ESHAP) and ifosfamide, carboplatin and etoposide (ICE) and local irradiation (3000 cGy). He was entered on this protocol, and on May 7, 1997 he underwent allogeneic bone marrow transplantation from his HLA-mismatched brother (2 of 6 HLA antigen mismatch [HLA-A and -B] in the GVH direction and one antigen mismatch [HLA-B] in the HVG direction). In the
30 second week post-transplant, an engraftment syndrome (fever and fluid retention) and grade II acute GVHD (skin and gastrointestinal tract involvement) developed, and responded promptly to corticosteroid therapy. A measurable decline in the size of his neck mass was evident immediately following chemotherapy. The mass subsequently regressed completely over a period of weeks following transplantation. Restaging at 100
35 days post-transplant confirmed that he was in a partial remission. Steroids were

discontinued approximately six months post-transplant, as the patient had only minimal cutaneous chronic GVHD. Low dose oral cyclosporine was continued. Approximately seven months post-transplant, an IgG warm antibody-mediated autoimmune hemolytic anemia and thrombocytopenia developed. Though initially responsive to oral corticosteroids, hemolytic anemia became exacerbated at 9 months, necessitating splenectomy, which showed no evidence of lymphoma upon pathological examination. The thrombocytopenia and hemolytic anemia resolved, and corticosteroid therapy was tapered. The patient is currently in complete remission from his lymphoma 15 months post-BMT, with no evidence of GVHD. Staging evaluations that included CT scans and bone marrow biopsies at seven months and one year post-transplant confirmed his complete remission status.

Because of the significant GVHD that developed in Patients 1 through 4, Patient 5, who was 51 years old, received a modified protocol that included less pre-transplant and more post-transplant ATG (15 mg/kg on days -1, +1, +3 and +5). Although grade II GVHD, manifested as fever, skin rash, and elevated liver enzymes, developed in the second week, the patient responded well to corticosteroids, which have been tapered without recurrence of GVHD. Staging at 100 days showed that he is in complete clinical remission.

Engraftment and establishment of mixed chimerism

These data are summarized for all five patients in Table 2. Leukopenia and thrombocytopenia occurred within 9 days post-BMT. Leukocyte engraftment (ANC > 500/mm³) occurred between 10 and 17 days post-BMT in all evaluable patients. Sustained recovery of platelets (to > 20,000/mm³) occurred at 9 to 72 days post-transplant in three patients. Patient 2 was still platelet transfusion-dependent at the time of her death on day 117. One patient died of pulmonary hemorrhage on day 12, before engrafting.

Chimerism was assessed by FCM analysis of WBC beginning 8 to 12 days post-BMT. All patients showed varying proportions of donor cells among lymphocytes, monocytes and neutrophils at the first time point tested (not shown), and at the time of last follow-up (Table 2). The time course for chimerism in these three lineages in Patient 1 is presented in Figure 1. Mixed chimerism has been sustained for at least one year, when 71% of monocytes, 62% of granulocytes, and 99% of lymphocytes were donor-derived (Figure 2). T cells and NK cells were > 98% donor-derived, whereas B cells were 33% host-derived and 67% donor-derived. Donor erythrocytes were

detectable beginning at 6 to 8 weeks post-BMT, and increased to about 80% of RBC by 9 months post-BMT. A marrow aspirate obtained one year post-BMT contained 73% donor and 27% host cells among the non-erythroid elements.

FCM analyses of Patients 1 through 4 relied on the use of HLA allele-specific mAbs that could specifically identify host, but not donor cells. The presence of donor cells, for which specific HLA allele-specific mAbs were not available, was verified in Patients 1, 2, and 4 by VNTR or STR analyses, which showed distinct donor bands in each sample.

10 DISCUSSION

Our studies demonstrate that lasting multilineage mixed hematopoietic chimerism, with high levels of donor reconstitution, can be induced across extensive HLA barriers in adult BMT recipients. Moreover, we show that such chimerism can be achieved in recipients conditioned with a non-myeloablative regimen, without severe or intractable GVHD, and that it is associated with striking anti-tumor responses in patients with advanced, refractory non-Hodgkins lymphomas.

Induction of mixed chimerism across MHC barriers has not, to our knowledge, been previously reported in adult humans. Lasting mixed chimerism in adults has been achieved across MHC barriers only in rodents^{14,15}. Mixed chimerism has been induced in dogs, but only in MHC-identical donor-recipient pairs¹⁶, and transient mixed chimerism has been achieved across MHC barriers in monkeys after non-myeloablative conditioning¹⁷.

The lack of myeloablation by the ATG/cyclophosphamide/thymic irradiation conditioning protocol used here is demonstrated by the survival of recipient hematopoietic progenitors that are capable of contributing to myeloid, lymphoid and erythroid lineages. Furthermore, in a similarly-conditioned recipient of an HLA-matched transplant (data not shown), secondary failure of donor hematopoiesis was not associated with significant cytopenias, due to the ability of surviving host hematopoietic progenitors to sustain multilineage hematopoiesis.

Transplantation of HLA-mismatched marrow in myeloablated humans has been associated with a significant incidence of failure of engraftment⁶, which can be reduced with the use of additional chemotherapy and immunotherapy^{18,19}. Our results suggest that specific targeting of host immune resistance with the combination of cyclophosphamide, anti-thymocyte globulin, and thymic irradiation can effectively

overcome host resistance to HLA-mismatched marrow engraftment, despite being less toxic than conventional myeloablative regimens.

In several recent studies of non-myeloablative conditioning regimens containing purine analogs in combination with other chemotherapeutic agents, high levels of donor reconstitution were achieved in recipients of HLA-matched sibling marrow. Although tumor responses were obtained primarily in patients with either conventional-risk malignancies or with chemosensitive disease in these studies, they are consistent with our own data showing that BMT can provide immunotherapy without host myeloablation²⁰⁻²². However, matched unrelated donor marrow failed to engraft in two of two patients in one of these studies²³, suggesting that the host conditioning may be less immunosuppressive than that used in our protocol, in which marrow mismatched at one or two of six HLA antigens in the host-vs-graft direction engrafted in all four cases.

Despite studies suggesting a graft-versus-lymphoma effect for allogeneic BMT in non-Hodgkins lymphomas, patients with chemoresistant disease have a very poor prognosis^{2,22,24-27}. Striking anti-tumor responses were achieved in several of our patients, despite the fact that they had very advanced, chemoresistant and even radioresistant, refractory disease. Two of the four evaluable patients are in complete and partial remissions at 460 and 103 days, respectively, and a third patient showed no evidence of disease progression at the time of death from aspergillosis on day 117. Since the only chemotherapy included in our protocol was cyclophosphamide, to which the patients all had prior exposure, the anti-tumor responses seen in our study implicate a potent immunotherapeutic effect of the donor marrow inoculum. Although longer follow-up and larger series of patients will be required to determine the potency and curative potential of this new approach to the treatment of non-Hodgkins lymphomas, the anti-tumor responses observed suggest that these may be superior to those achievable with conventional lethal TBI/cyclophosphamide and HLA-matched or closely-matched BMT. This might occur because of the more potent alloresponses directed against MHC alloantigens than against minor histocompatibility antigens, which have led to enhanced GVL effects in rodent studies^{3,4}. In addition, the presence of host-derived professional antigen-presenting cells in mixed chimeras could be associated with enhanced GVL effects for other reasons, perhaps related to their ability to efficiently present host alloantigens that are shared by tumor cells.

The more potent alloresponses generated against MHC disparities compared to those against minor histocompatibility antigens usually elicits severe GVHD, which has been the major impediment to HLA-mismatched BMT⁵. The patients described here developed GVHD, but in several patients it was surprisingly mild and amenable to

corticosteroid therapy. GVHD prophylaxis consisted of cyclosporine plus a single post-transplant (day +1) treatment with ATG in four patients, and in the fifth patient, whose GVHD was also well-controlled with corticosteroids, an increased proportion of the conditioning ATG was given post-transplant rather than pre-transplant. Less severe host conditioning^{28,29} and the initial presence of host hematopoietic elements^{30,31} have both been shown to reduce the severity of GVHD in rodents. Larger patient series will determine whether or not our new regimen will allow the routine performance of HLA-mismatched BMT without unacceptable GVHD.

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10

(v) Other Embodiments

As an alternative or adjunct to hemoperfusion, host antibodies can be depleted by administration of an excess of hematopoietic cells.

- 15 Stromal tissue can be introduced prior to hematopoietic cell transplant, e.g., BMT. It may be varied by: (1) administering the fetal liver and thymus tissue as a fluid cell suspension; (2) administering fetal liver or thymus stromal tissue but not both; (3) placing a stromal implant into other encapsulated, well-vascularized sites, or (4) using adult thymus or fetal spleen as a source of stromal tissue.

- 20 An anti-CD2 antibody, preferably a monoclonal, e.g., BTI-322, or a monoclonal directed to a similar or overlapping epitope, can be used in addition to or in place of any anti-T cell antibodies (e.g., ATG) in any method referred to herein.

Methods of preparing the recipient for transplant of hematopoietic stem cells may be varied. For instance, recipient may undergo a splenectomy. The latter would preferably be administered prior to the non-myeloablative regimen, e.g., at day -14.

(vi) Incorporation by reference

All of the above-cited references and publications are hereby incorporated by reference.

5

(vii) Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following

10 claims.

Other embodiments are within the following claims.

What is claimed is:

1. A method of treating a subject having a hematologic disorder comprising:
administering a myeloreductive non-myeloablative treatment to the
subject in sufficient amount such that mixed hematopoietic chimerism can be
induced in the subject, and
introducing into the subject, allogeneic donor hematopoietic stem cells
(donor stem cells) to form chimeric bone marrow in the subject.
2. The method of claim 1, wherein the myeloreductive treatment includes treating
the subject with an immunosuppressant regimen, prior to introduction of the
donor stem cells, in an amount sufficient to prevent rejection of the donor stem
cells.
3. The method of claim 1 or 2, comprising the further step of treating the subject
with an immunosuppressant regimen, after introduction of the donor stem cells,
in an amount sufficient to prevent a graft-versus-host response mediated by the
donor cells and to prevent rejection of the donor stem cells.
4. The method of claim 2 or 3, wherein the immunosuppressant regimen includes
inactivating or depleting host T-lymphocytes and/or natural killer (NK) cells in
the subject.
5. The method of claim 2, 3 or 4, wherein the immunosuppressant regimen includes
treatment with T cell-depleting anti-CD4 and/or CD8 antibodies.
6. The method of claim 5, wherein the immunosuppressant regimen includes
treatment with anti-thymocyte globulin (ATG).
7. The method of claim 5, wherein the immunosuppressant regimen includes
treatment with one or more of OKT3, LO-CD2a, Minnesota anti-lymphoblast

globulin (MALG)

8. The method of claim 2, 4 or 5, wherein the immunosuppressant regimen includes treatment with thymic irradiation.
- 5
9. The method of claim 3, 4 or 5, wherein the immunosuppressant regimen includes treatment with sub-lethal nonmyeloablative irradiation of lymphocyte-containing tissue, a costimulatory blocking agent.
- 10
10. The method of claim 1, 2 or 3, wherein the myeloreductive treatment further includes treating the subject, prior to introduction of the donor stem cells, with an cytoreductive agent selected from one or more of alkylating agents (e.g., nitrogen mustards [such as mechlorethamine], cyclophosphamide, melphalan and chlorambucil), alkyl sulphonates (e.g., busulphan), nitrosoureas (e.g., carmustine, lomustine, semustine and streptozocine), triazenes (e.g., dacarbazine), antimetabolites (e.g., folic acid analogs such as methotrexate),
15 pyrimidine analogs (e.g. fluorouracil and cytarabine), purine analogs (e.g., fludarabine, idarubicin, cytosine arabinoside, mercaptopurine and thioguanine), vinca alkaloids (e.g., vinblastine, vincristine and vendesine), epipodophyllotoxins (e.g., etoposide and teniposide), antibiotics (e.g., dactinomycin, daunorubicin,
20 doxorubicin, bleomycin, plicamycin and mitomycin), dibromomannitol, deoxyspergualine, dimethyl myleran and thiotepa.
11. The method of claim 6, 8 or 10, wherein the myeloreductive treatment includes treating the subject with cyclophosphamide.
- 25
12. The method of claim 1, 2, 3, 8 or 10, wherein the donor stem cells are mismatched, with respect to the subject, at one or more class II HLA antigens.
13. The method of claim 1, 2, 3, 8, 10 or 12, wherein the donor stem cells are mismatched, with respect to the subject, at two or more HLA antigens.
30
14. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are provided as allogeneic bone marrow.

15. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are provided as mobilized peripheral blood cells.
- 5 16. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are provided as cord blood cells.
17. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are provided as *ex vivo* expanded stem cells.
- 10 18. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are from the same species as the subject.
- 15 19. The method of claim 1, 2, 3, 10, 12 or 13, wherein the donor stem cells are xenogeneic stem cells from a different species than the subject.
20. The method of claim 1, 2, 3, 10, 12 or 13, wherein the subject is a human.
- 20 21. The method of claim 1, 2, 3, 10, 12, or 20, wherein the hematologic disorder includes neoplastic proliferation of hematopoietic cells.
22. The method of claim 21, wherein the hematologic disorder is a leukemia.
- 25 23. The method of claim 21, wherein the hematologic disorder is selected from the group consisting of lymphoblastic leukemia, acute or chronic myelogenous leukemia, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, myelodysplastic syndrome, multiple myeloma, and chronic lymphocytic leukemia.
- 30 24. The method of claim 21, wherein the hematologic disorder is refractory to chemotherapy.

25. The method of claim 24, wherein the hematologic disorder is chemorefactory Non-Hodgkin's lymphoma.
- 5 26. The method of claim 1, 2, 3, 10, 12, or 20, wherein the hematologic disorder is a non-malignant disorder.
27. The method of claim 26, wherein the hematologic disorder is an inherited erythrocyte abnormalities or inherited immune system disorders.
- 10 28. The method of claim 26, wherein the hematologic disorder is a hemoglobinopathy, e.g., sickle cell anemia, aplastic anemia or thalassemia.
- 15 29. The method of claim 1, 2, 3, 10, 12, or 20, comprising the further step of administering allogeneic donor leukocytes to the subject after introduction of the donor stem cells.
30. A method of treating a subject having a hematologic disorder comprising:
administering a myeloreductive and immunosuppressive treatment to the
20 subject in sufficient amount such that mixed hematopoietic chimerism can be induced in the subject, and
introducing into the subject, allogeneic donor hematopoietic stem cells (donor stem cells) to form stable mixed chimeric bone marrow in the subject.
- 25 31. A method of treating a patient having neoplastic hematopoietic disorder, comprising:
identifying a patient having a neoplastic hematopoietic disorder,
administering a myeloreductive non-myeloablative treatment to the
subject in sufficient amount such that macroscopic mixed chimerism can be
30 induced in the subject, and
introducing into the subject, allogeneic donor hematopoietic cells (donor

stem cells) to form chimeric bone marrow in the subject and induce a graft-versus-leukemia response and/or graft-versus-lymphoma response, which donor stem cells are mismatched, with respect to the patient, at one or more HLA-A, B or DR antigens.

5

32. The use of donor allogeneic hematopoietic cells in the manufacture of a medicament for the treatment of a hematologic disorder, said medicament administered to a patient conditioned with myeloreductive non-myeloablative treatment, and in an amount sufficient to form chimeric bone marrow in the subject.

10

33. The method of claim 12, wherein the donor stem cells are mismatched, with respect to the subject, at an HLA-DR antigen.

15

34. The method of claim 13, wherein the donor stem cells are mismatched, with respect to the subject, at two or more HLA-A, B or DR antigens.

35. The method of claim 29, wherein the allogeneic donor leukocytes are administered at least 14 days after transplantation.

20

36. The method of claim 29 or 35, wherein the subject is tested for GVHD, and the allogeneic donor leukocytes are administered if no GVHD is evident.

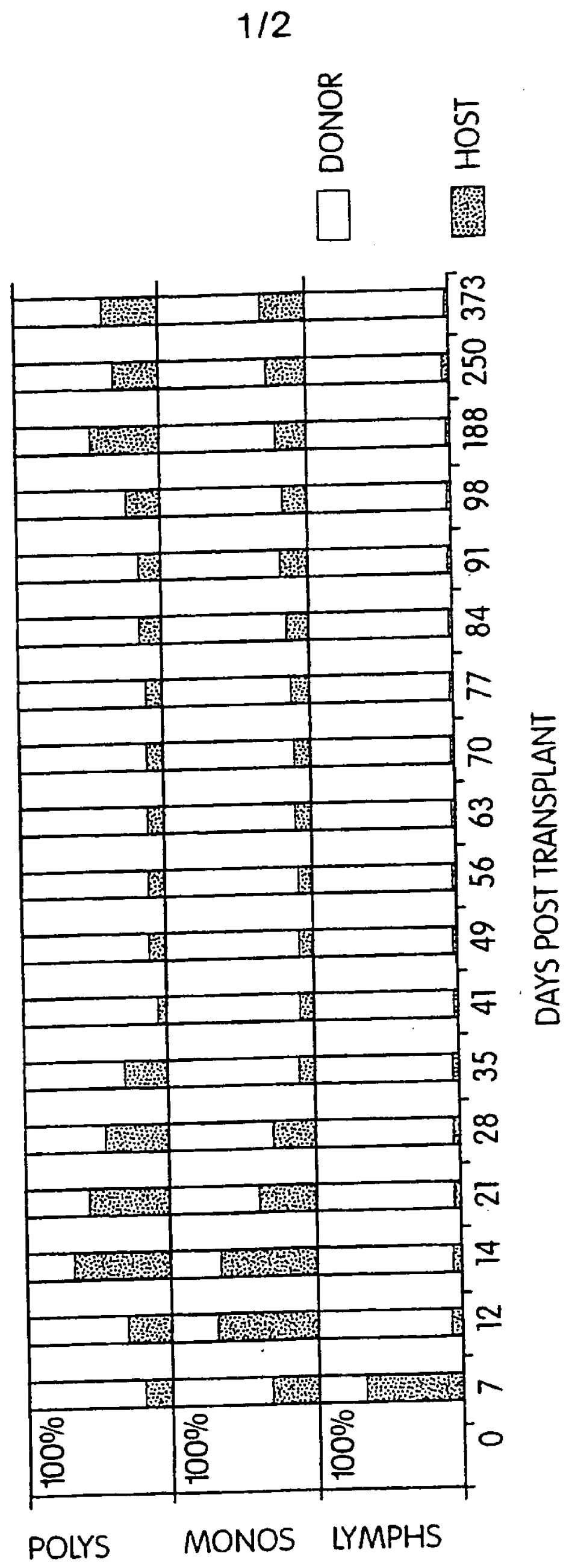
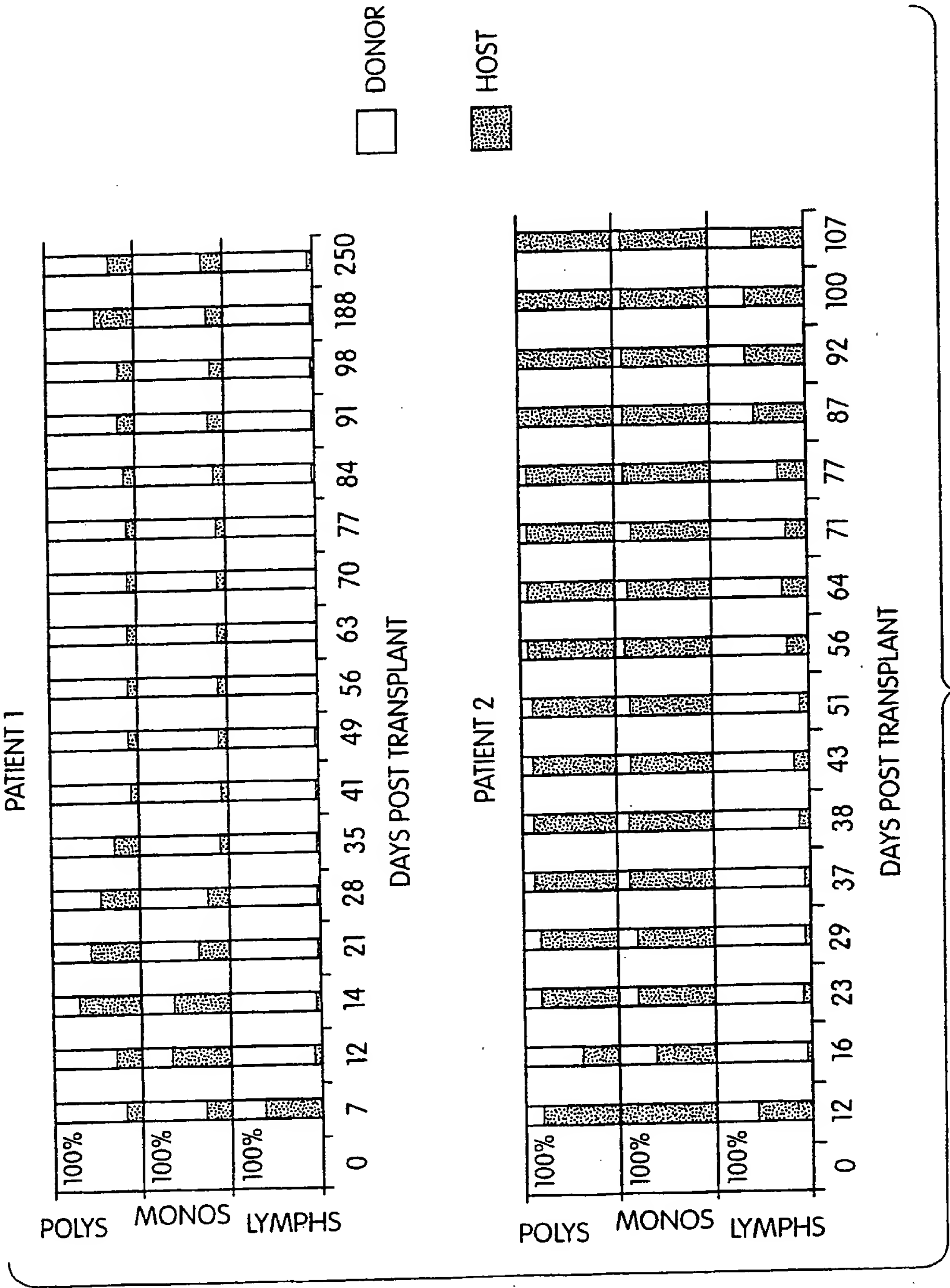


Fig. 1



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 1-31, 33-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

Claims Nos.: 1-31, 33-36

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/24209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	SLAVIN S. ET AL: "Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with letal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases" BLOOD, vol. 91, no. 3, 1 February 1998, pages 756-763, XP002101607 see the whole document	1-36
A	WO 89 04168 A (BIOCYTE CORP) 18 May 1989 see the whole document	1-36

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INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/US 98/24209

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/14 A61K35/28 //(A61K35/28, 39:395, A61K31:675)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NIKOLIC B. ET AL: "Bone marrow chimerism and transplantation tolerance" CURRENT OPINION IN IMMUNOLOGY, vol. 9, no. 5, 1997, pages 634-640, XP002101603 see abstract see page 635, "T cell tolerance and mixed bone marrow chimerism" see page 638, column 1, paragraph 2 see page 638 "conclusions" ---	1-36
X	US 5 635 156 A (ILDSTAD SUZANNE T) 3 June 1997 see column 3, line 23 - line 60 see column 4, line 63 - line 67 see column 5, line 1 - line 39 see column 9, line 47 - line 55 see column 11, line 47 - line 56 --- -/-	1-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

29 April 1999

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/24209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIRALT S. ET AL: "Engraftment of allogenic hematopoietic progenitor cells with purine analog-containing chemotherapy: Harnessing graft-versus-leukemia without myeloablative therapy" BLOOD, vol. 89, no. 12, June 1997, pages 4531-4536, XP002101604 see abstract see page 4531, column 1 see page 4534-4535 "discussion" ---	1-36
X	WO 93 13785 A (GEN HOSPITAL CORP) 22 July 1993 see page 10, line 28 - page 13, line 6 see claims 1,2,11,15 ---	1-36
X	WO 95 03062 A (CELLPRO INC) 2 February 1995 see page 3, line 26 - line 32 see page 11, line 26 - page 12, line 11 see page 16 - page 17 ---	1-36
X	WO 97 41863 A (GEN HOSPITAL CORP ; SYKES MEGAN (US)) 13 November 1997 see examples 1,2 see page 32 - page 34 see page 35, line 21 - line 24 ---	1-36
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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/24209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	SLAVIN S. ET AL: "Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with letal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases" BLOOD, vol. 91, no. 3, 1 February 1998, pages 756-763, XP002101607 see the whole document	1-36
A	WO 89 04168 A (BIOCYTE CORP) 18 May 1989 see the whole document	1-36

INTERNATIONAL SEARCH REPORT

International application No

PCT/US 98/ 24209

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-31, 33-36
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 1-31, 33-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

Claims Nos.: 1-31, 33-36

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 35/14, 35/28 // (A61K 35/28, 39:395, 31:675)		A3	(11) International Publication Number: WO 99/25367 (43) International Publication Date: 27 May 1999 (27.05.99)
(21) International Application Number: PCT/US98/24209 (22) International Filing Date: 13 November 1998 (13.11.98) (30) Priority Data: 60/073,230 14 November 1997 (14.11.97) US (71) Applicant (for all designated States except US): THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SYKES, Megan [US/US]; 197 Eighth Street #301, Boston, MA 02129 (US). SPITZER, Thomas, R. [US/US]; 14 Seten Circle, Andover, MA 01810 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, One Post Office Square, Boston, MA 02109 (US).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 5 August 1999 (05.08.99)
(54) Title: TREATMENT OF HEMATOLOGIC DISORDERS (57) Abstract The inventors have discovered that hematologic disorders, e.g., both neoplastic (hematologic cancers) and non-neoplastic conditions, can be treated by the induction of mixed chimerism using myeloreductive, but not myeloablative, conditioning. Methods of the invention reduce GVHD, especially GVHD associated with mismatched allogeneic or xenogeneic donor tissue, yet provide, for example, significant graft-versus-leukemia (GVL) effect and the like.			

INTERNATIONAL SEARCH REPORT

Information on patent family members

In. tional Application No

PCT/US 98/24209

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Paediatric Rheumatology Workshop/Series Editor: P. Woo

The depletion of T cells from haematopoietic stem cell transplants

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CLB, Sanquin Blood Supply Foundation, Department of Transfusion Technology, Stem Cell Laboratory, Laboratory for Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam, ¹Department of Internal Medicine, Academic Medical Centre, Amsterdam, ²Department of Haematology, University Hospital Utrecht, Utrecht, ³Department of Paediatric Immunology, University Hospital for Children, 'het Wilhelmina Kinderziekenhuis', Utrecht and ⁴Department of Paediatrics, University Hospital Leiden, Leiden, The Netherlands

Abstract

Objective. In our laboratory, we have developed an immunorosette technique for the depletion of T cells from bone marrow transplants. Tetrameric complexes of monoclonal antibodies are able to form very stable immunoroses, which are efficiently depleted with the aid of a blood cell separator. Major improvements over the original sheep red blood cell depletion are the use of human (patient or donor derived) erythrocytes instead of sheep-derived cells, and the possibility of using a closed system for separation in a cell separator. In contrast to bone marrow, mobilized haematopoietic stem cell transplants obtained after leucocytapheresis contain higher numbers of T cells. Therefore, a different approach is necessary.

Method. We have used two CD34 selection systems (Isolex[®] 300SA and the Clinimacs[®]) to perform T-cell depletions from peripheral blood stem cell (PBSC) transplants.

Results. Immunorosette T-cell depletion, with CD2/CD3 tetrameric complexes, of bone marrow transplants resulted in a mean 2.5 log depletion of T cells with a yield of 50% of the CD34⁺ cell population. Stem cell selection of PBSC transplants using one of the CD34 selection procedures resulted in a 4.5 log depletion of T cells for both systems, but with different results for the recovery of CD34⁺ cells. An increased yield of CD34⁺ cells was obtained with the Clinimacs[®] procedure ($57.9 \pm 9.0\%$) in comparison to the Isolex[®] procedure ($40.1 \pm 12.5\%$).

Conclusion. Our own immunorosette depletion technique and the two tested CD34 selection methods for stem cell transplants both resulted in a very efficient T-cell depletion with the recovery of 40-60% of the CD34⁺ haematopoietic stem cells present in the transplant.

KEY WORDS: T cells, Depletion, Haematopoietic stem cell transplants.

In the past, several different T-cell depletion techniques have been developed to avoid graft-vs-host disease in an allogeneic stem cell transplantation setting. Kernan *et al.* [1] have applied an agglutination technique using soy

bean agglutinin (SBA) followed by T-cell depletion using sheep red blood cells (SRBC). This method was the first and one of the most widely applied T-cell depletion techniques for the removal of T cells from bone marrow. Others have performed methods, for instance, based on the difference in cell size between T cells and haematopoietic stem cells, known as the counterflow elutriation technique [2], or antibody-mediated techniques, like complement-mediated cell lysis using Campath antibody [3].

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In our institute, Lansdorp *et al.* [4] developed a technique using bivalent antibody complexes, known as tetrameric complexes. We have modified the application of this method and introduced an immunorosette procedure to couple the patient's own erythrocytes to target cells for efficient depletion. Originally, this technique was set up to deplete malignant cells from patients with B-cell malignancies [5], but we have also produced tetrameric complexes for the removal of T cells. This technique can now be used to deplete T cells from allogeneic or autologous bone marrow stem cell transplants.

Since peripheral blood stem cells (PBSC) are rapidly replacing bone marrow as a source of haematopoietic stem cells, other techniques have been developed for the removal of T cells. PBSC transplants from normal donors contain higher percentages of T cells, and often > 10 times the total number of T cells. This necessitates another approach. CD34 cell selection is an efficient method to achieve T-cell removal. Several different methods for clinical application are commercially available with variable results. Cellpro was the first on the market with a Food and Drug Administration-approved column system (Ceprate^R SC) using biotin-labelled CD34 monoclonal antibody (MAb) in combination with avidin-coated Sepharose beads. This selection procedure results in a 40–50% yield of CD34 stem cells with a purity of ~80%. Then, Baxter introduced the Isolex[®] system for positive selection, using Dynabeads^R to isolate the stem cells and a CD34-releasing peptide (PR 34+[®]) to dissolve the bond between the stem cells and the beads. This method also results in a 40–50% yield of CD34⁺ cells, but with a much higher purity of >90% CD34⁺ cells. Recently, Miltenyi Biotec has introduced an immunomagnetic system, Clinimacs[®], using very small beads (colloidal super paramagnetic MicroBeads), which can efficiently enrich the stem cell population (>90% pure) like the Isolex[®] system, but with a higher CD34 yield of $\pm 65\%$ [6]. Using this system, the beads, which are very small, are not removed from the surface of the CD34⁺ stem cells.

Autologous stem cell transplants have recently been introduced for the treatment of patients with autoimmune diseases [7]. Most of these diseases are believed to be T-cell mediated, so a depletion of autologous T cells seems indicated. However, an exact T-cell dose is not yet known, but a more extensive reduction than 1×10^5 T cells/kg body weight (BW) might not be necessary (van Bekkum [8]).

In this article, we present an outline of our results obtained with immunorosettes for the depletion of T cells from bone marrow, and CD34 selection for the depletion of T cells from PBSC.

Materials and methods

Allogeneic stem cell transplants

Patients suffering from several different haematological malignancies, who have an allogeneic donor available,

were treated with high-dose chemotherapy and total body irradiation. On the day of transplant, bone marrow cells were taken from either HLA-identical siblings or matched unrelated donors, and sent to the cell-processing laboratories of the CLB in Amsterdam, or of the Academic Hospital in Utrecht. More recently, the use of PBSC transplants for rapid haematopoietic reconstitution in an HLA-identical setting has become more beneficial for the patients than using bone marrow as a source of stem cells. Donors were given 5 μ g granulocyte-colony stimulating factor (G-CSF) for mobilization of PBSC. The leucocytapheresis procedures were performed at the Academic Medical Centre. The PBSC transplants were transported to the CLB and either CD34 selected directly or the next day. After selection, the cells were taken to the hospital and directly infused.

Autologous stem cell transplants

Autologous bone marrow transplants were harvested from children with juvenile chronic arthritis, according to a protocol described in this issue by Wulffraat and Kuis [9].

Techniques for T-cell depletion

Immunorosette depletion of bone marrow. Tetrameric complexes are formed by the addition of cross-linking RaMIgG1 MAb to a mixture of MAbs, one directed against glycophorin A in the membrane of human erythrocytes and another T-cell-specific MAb (CD2 or CD3). These complexes are then bound to erythrocytes and the coated erythrocytes are washed. The bone marrow harvest is centrifuged, prior to depletion, and a buffy coat suspension is prepared to get rid of the excess erythrocytes. After addition of the coated erythrocytes to the bone marrow buffy coat cells, immunorosettes are formed. These immunorosettes are depleted using a Ficoll density separation ($d = 1.077 \text{ g/cm}^3$) in an IBM 2991 cell processor and the light density cells are washed and cryopreserved.

CD34 selection of PBSC. In our laboratory, we have used two different CD34 selection systems for the removal of T cells from PBSC transplants: Isolex[®] 300SA (Baxter Biotech group, USA) and the Clinimacs[®] (Miltenyi Biotec, Germany). Procedures were performed according to the manufacturers' instructions.

Quality control

The quality of the transplant was measured using the myeloid progenitor cell assay (CFU-GM, colony forming unit for granulocytes and monocytes) with human placental conditioned medium as a source of growth factors, and by monitoring the CD34 content. The efficacy of the T-cell depletion technique was measured either by means of the immunorosette method or by immunofluorescence using the FACscan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Results

Immunorosette depletion

The immunorosette technique was successfully applied to allogeneic bone marrow transplants from HLA-identical siblings (Table 1).

The target number of T cells to be transplanted at the Academic Medical Centre was set at 1×10^5 T cells/kg BW, according to the protocol described by Verdonck *et al.* [10]. This protocol aims to minimize the risk of graft-vs-host disease and inducing a graft-vs-leukaemia effect in patients with haematological malignancies.

In all CD2/CD3-depleted stem cell transplants, a supplement of unseparated bone marrow cells was necessary to reach 1×10^5 T cells/kg BW. This enabled transplantation of exact numbers of T cells in these patients. In four patients, the recovery of the CD34 haematopoietic stem cell population was measured and the CD34 yield was $49.1 \pm 18.8\%$.

Furthermore, we have used the same technique to reduce the number of T cells in autologous bone marrow transplants from children with juvenile chronic arthritis (Fig. 1). Here, the aim was to reach an even lower level of T cells: 1×10^4 T cells/kg BW. By performing a second round of T-cell depletion, this target number was reached in two out of six autologous transplants, with an average of $5.1 \pm 4.9 \times 10^4$ T cells/kg BW for all six patients.

The mean CD34 recovery was $53.5 \pm 27.5\%$, which

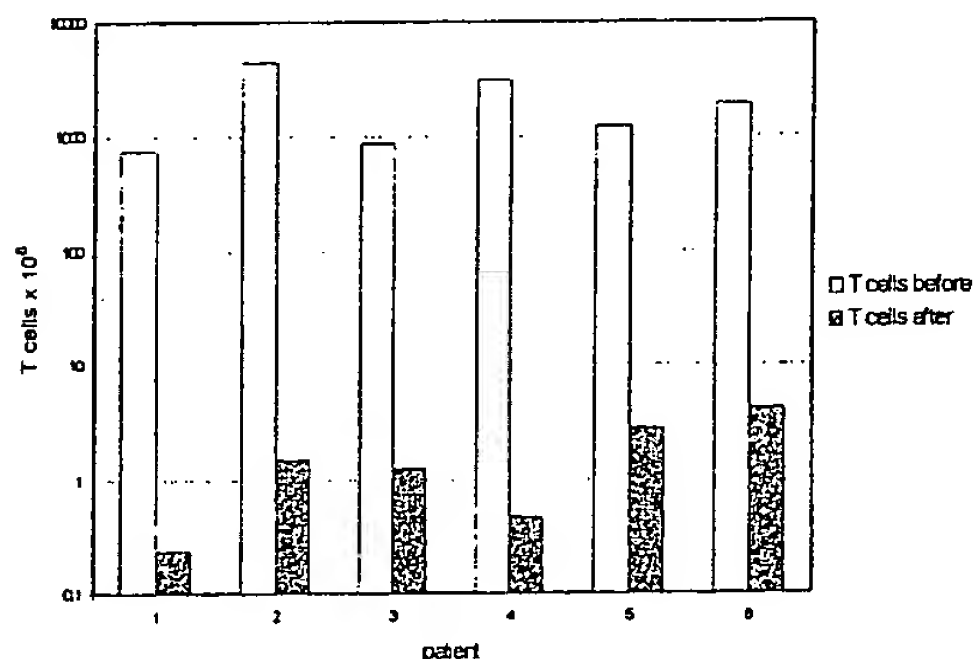


FIG. 1. Results of the depletion of CD2 and CD3 immunorosetted T cells from bone marrow transplants of children with juvenile chronic arthritis. Depicted are the total T-cell numbers in the bone marrow harvest (T cells before) and total T-cell numbers after depletion.

allowed the infusion of $1.5 \pm 1.1 \times 10^6$ CD34⁺ cells/kg BW (range 0.5–3.6).

In Utrecht, the same CD2 and CD3 immunorosette procedure was performed on 34 allogeneic bone marrow transplants, resulting in a 2.3 log depletion of T cells (Fig. 2).

CD34 selection

For the depletion of T cells from PBSC transplants, we have used two different CD34 selection systems: the Isolex[®] and Clinimacs[®]. The average depletions of T cells from PBSC transplants for both procedures are equal: 4.5 logs (Fig. 2). However, there is a great difference in the recovery of CD34⁺ cells: the Isolex[®] procedure resulted in a median recovery of $40.1 \pm 12.5\%$, while the Clinimacs[®] procedure resulted in a $57.9 \pm 9.0\%$ recovery of CD34⁺ cells.

Discussion

The main advantage of using the immunorosette depletion technique instead of the SBA/SRBC procedure is the fact that it can be performed in a closed system. Very stable immunorosettes are formed, which can easily be separated in the IBM cell processor. Furthermore, binding of the patient's own or donor-derived erythrocytes to the T cells circumvents the use of SRBC, which cannot be produced according to good manufacturing practice regulations. In our procedures, MAbs were used that were screened according to the CLB Biosafety Testing protocols, in the absence of bacterial and viral contamination. Moreover, combining the removal of immunorosettes within one round of density separation

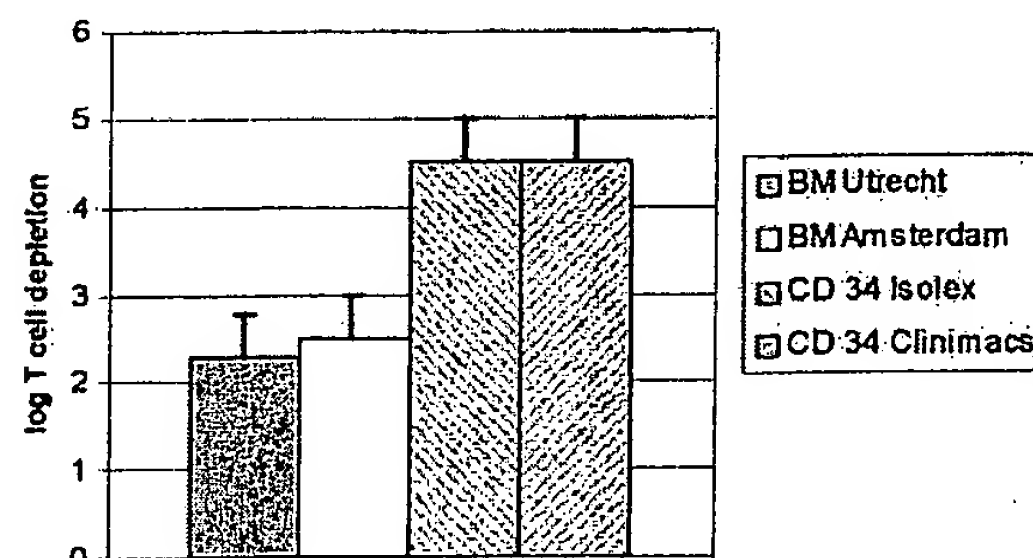


FIG. 2. T-cell depletion results of bone marrow and PBSC transplants obtained with the different methods. Bone marrow processing was performed at two centres.

TABLE 1. Results of the T-cell reduction in bone marrow transplants of HLA-identical donors.

	Cell number $\times 10^8$	CFU-GM $\times 10^4$	CD3 ⁺ cells $\times 10^6$
Bone marrow harvest (n = 10)	176.8 ± 45.3	1910 ± 749	2769 ± 719
CD2/CD3 depleted (n = 10)	10.19 ± 4.27 (5.8 \pm 2.4%)	780 ± 419 (50.9 \pm 19.4%)	0.55 ± 0.90 (0.022 \pm 0.045%)

The recovery of each cell population is given in parentheses.

reduced the processing time from 9 to 6 h, thereby saving ~50% of the CD34⁺ cells. Using immunorosettes for the depletion of T cells from bone marrow, a significant removal of T cells occurred, averaging a 2.5 log T-cell depletion.

So far, we have only limited experience with the isolation of CD34⁺ cells from bone marrow ($n = 2$), with varying results: 6% yield with the Isolex[®] system and 50.2% with the Clinimacs. Apart from the prolonged selection procedure (density separation followed by selection), the CD34 selection methods are all very expensive. This method is not only costly because of the separation device, but also because the disposables, media and CD34 kits are expensive.

For PBSC transplants, however, we have so far tested both immunomagnetic systems of Baxter and Miltenyi. In a recently published study [11], the CD34 selection systems of Cellpro and Baxter were compared, resulting in a 3.4 median log T-cell depletion for the Isolex[®] 300I system and a 2.9 log T-cell depletion for the Cephate[®] system.

Our own results indicate that similar T-cell depletion efficacies are being reached using the two immunomagnetic selection systems Isolex[®] 300SA (Baxter) and the Clinimacs[®] (Miltenyi).

In our laboratory, we are currently performing experiments to develop new methods using the tetrameric complexes for depletion of larger numbers of T cells in combination with a nylon wool filtration technique as described in part by Kwekkeboom *et al.* [12] so that we can perform a much cheaper technique than the CD34 selection for PBSC.

Moreover, we think that CD34 selection is not the optimal method for T-cell depletion, since all other cell types are also excluded from the transplant, including cells which, in an allogeneic setting, might play a role in the engraftment of the haematopoietic stem cells. Further studies will indicate whether we can successfully develop a T-cell depletion technique for PBSC transplants.

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